

**Investigation of the Function of Disproportionating
Enzyme in Potato (*Solanum tuberosum* L.)**

Juliette Elizabeth Snow

Doctor of Philosophy

The University of Edinburgh

2000



Abstract

Disproportionating enzyme (D-enzyme, 4- α -glucanotransferase, EC 2.4.1.25) catalyses the breakage and reformation of α -1,4 links in 1,4- α -D-glucans. The enzyme catalyses glucan transfer from one α -1,4-glucan molecule to another, to glucose or to itself to form cyclic molecules. The preferred substrates of the enzyme *in vitro* are malto-oligosaccharides (MOS). In potato, D-enzyme is present in leaves throughout the diurnal cycles of light and dark but is particularly abundant in tubers. From analysis of transgenic potato plants with lowered D-enzyme activity and a D-enzyme knock-out mutant of *Arabidopsis thaliana*, evidence has been found to support a role for D-enzyme in starch degradation. However, a D-enzyme mutant from *Chlamydomonas reinhardtii* has provided evidence that D-enzyme could also be involved in starch synthesis. In support of this, D-enzyme mRNA has been found to accumulate during periods of starch synthesis in many species including potato. Potentially then, D-enzyme could be involved in starch synthesis, degradation or both.

In this study potato plants with lowered D-enzyme activity were investigated. It was found previously that lack of D-enzyme resulted in a reduction in the tuber yield of these plants and a delay in tuber sprouting. In this study the reduction in tuber yield was characterised further. It was found that tuber fresh weight per plant was reduced but percentage dry matter and starch content were unaltered. In addition, the extent of the reduction in yield was dependent on irradiance. Lack of D-enzyme also resulted in a delay in sugar accumulation in tubers during prolonged storage. No differences were detected in rates of starch synthesis and starch turnover in tubers with lowered D-enzyme compared to controls. One reason for this could have been that the labelling experiments employed to investigate tuber metabolism were not sensitive enough to detect small differences. To address this, D-enzyme activity was lowered in transgenic potato plants which exhibit exaggerated rates of starch synthesis and turnover due to the expression of a heterologous ADPglucose pyrophosphorylase gene. It was hypothesised that differences in starch metabolism would be more likely to be resolved in tubers from these plants. Surprisingly, no appreciable differences between the starch metabolism of tubers with exaggerated rates of starch turnover and lowered D-enzyme activity and control tubers were detected. However, the level of D-enzyme activity in these tubers was 14 % of wild type and this could have been high enough for the *in vivo* role(s) of the enzyme to be fulfilled. Rates of starch breakdown in leaf tissue lacking D-enzyme were slightly reduced compared to controls during darkness. Lack of D-enzyme did not affect rates of starch synthesis during the light. These results are consistent with those from the D-enzyme mutant of *A. thaliana* and suggest that D-enzyme may have a role in potato leaf starch degradation during darkness and that this could in turn influence tuber growth.

Acknowledgements

I would like to thank everyone at Advanced Technologies (Cambridge) Ltd. and lab 205, Rutherford Building, The University of Edinburgh who was involved in my project. In particular, Dr Jutta Tuerck, Dr Steve Coates, Ms Susie Davenport, Dr Dawn Carter, Dr Sarah Sherson and Dr Georg Hemmann.

I would like to thank Dr Takeshi Takaha, Glico Co., Japan for his rapid responses to my many queries. I would also like to thank Dr A. M. Smith and Dr S. Zeeman, John Innes Institute, Norwich for allowing me to spend time in their lab to learn the MOS protocol.

I am extremely grateful for the support and encouragement I have received over the last three years from my supervisors Dr M. M. Burrell, Advanced Technologies (Cambridge) Ltd., and Dr S. M. Smith, The University of Edinburgh. I would like to thank the BBSRC and Advanced Technologies (Cambridge) Ltd. for funding my CASE studentship. I also want to thank my parents for their colossal support throughout my education.

Lastly, I want to say thank you to Pete for his patience and support whilst I was writing my thesis and more importantly for dragging me away to the mountains at every possible opportunity.

Abbreviations and Glossary

(v/v)	volume:volume ratio
(w/v)	weight:volume ratio
3-PGA	3-phosphoglycerate
°C	degree Celsius
[U- ¹⁴ C]	¹⁴ C universally labelled carbon
A	absorbance
ADP	adenosine 5'-diphosphate
ADP-glc	ADP-glucose
AGPase	ADP-glucose pyrophosphorylase
amp	ampicillin
ANOVA	analysis of variance
APS	ammonium persulphate
<i>A. tumefaciens</i>	<i>Agrobacterium tumefaciens</i>
ATP	adenosine 5'-triphosphate
BAP	6-benzylaminopurine
BE	branching enzyme
bp	base pairs
Bq	Becquerel
BSA	bovine serum albumin
Bt 1	Brittle 1
CaMV	Cauliflower mosaic virus
cDNA	complementary DNA
Ci	Curie
Cla	claforan
c.p.m.	counts per minute
C-terminal	carboxy terminal
cv.	cultivar
2,4-D	2,4-dichlorophenoxyacetic acid
Da	dalton
DBE	debranching enzyme
DHPS	dihydropteroate synthase
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
dNTPs	deoxynucleoside triphosphates
DP	degree of polymerisation
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EtOH	ethanol
GA3	gibberellic acid
G-1-P	glucose-1-phosphate
G-6-P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
GBSS	granule-bound starch synthase
<i>glgC-16</i>	<i>E. coli</i> AGPase gene
<i>gus</i>	β-glucuronidase gene
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
kan	kanamycin
LB	Luria-Bertani medium
Mr	relative molecular mass
mRNA	messenger RNA

MS	Murashige and Skoog medium
MSG	MS 3% glucose liquid medium
N-terminal	amino terminal
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)
<i>npt II</i>	aminoglycoside phosphotransferase encoding gene
Ω	ohms
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
Pho1	plastidic-type of phosphorylase
Pho2	cytosolic-type of phosphorylase
Pi	orthophosphate
PolyA	polyadenylation
PPi	pyrophosphate
rif	rifampicin
RNA	ribonucleic acid
RNAase	ribonuclease
r.p.m.	revolutions per minute
s.e.m.	standard error of the mean
SDS	sodium dodecyl sulphate
SS	starch synthase
SSS	soluble starch synthase
strep	streptomycin
<i>sul</i>	sulphonamide resistance gene
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TBE	Tris-borate EDTA
T-DNA	transfer DNA
TEMED	N,N,N',N'-tetramethyl ethylenediamine
Ti	tumour inducing
TRIS	2-amino-2-hydroxymethylpropane-1,3-diol
u	unit
μ FD	microfarad
UV	ultraviolet
V	volt
<i>vir</i>	<i>A. tumefaciens</i> virulence genes

Contents

Chapter 1 Introduction	1
1.1 The potato	1
1.2 Starch	1
1.2.1 Occurrence and uses	1
1.2.2 Components of starch	2
1.2.3 Starch structure	2
1.2.4 Transitory and storage starch	5
1.3 Starch Synthesis	5
1.3.1 Overview	5
1.3.2 Regulation of starch synthesis	7
1.3.3 Mechanism of starch granule synthesis	7
1.3.3.1 <i>Amylose</i>	7
1.3.3.2 <i>Amylopectin</i>	9
1.3.4 Models of amylopectin synthesis	11
1.4 Starch degradation	13
1.4.1 Enzymes of starch degradation	13
1.4.2 Starch degradation in photosynthetic organs	16
1.4.3 Starch degradation in storage tissues	17
1.4.4 Source-sink relations in potato	18
1.5 D-enzyme	19
1.5.1 Occurrence of D-enzyme	19

1.5.2	Reactions catalysed by D-enzyme	20
1.5.2.1	<i>Disproportionation</i>	20
1.5.2.2	<i>Cyclisation</i>	20
1.5.3	Localisation and expression	21
1.5.4	The function of D-enzyme	21
1.5.4.1	<i>Mammalian glycogen metabolism</i>	21
1.5.4.2	<i>Microbial 4-α-glucanotransferases</i>	22
1.5.4.3	<i>Role of D-enzyme in starch metabolism</i>	24
1.6	Project aims	28
2	Chapter 2 Materials and Methods	29
2.1	Materials	29
2.1.1	Plant material	29
2.1.2	Biochemical reagents and equipment	29
2.1.3	Molecular biology materials	30
2.1.4	SDS-PAGE reagents and materials	30
2.1.5	Bacteriological and plant media	31
2.2	Biochemical procedures	32
2.2.1	Sampling of tissue	32
2.2.2	Spectrophotometric analysis	32
2.2.3	Enzyme activity assays	33
2.2.3.1	<i>Preparation of enzyme extract</i>	33
2.2.3.2	<i>D-enzyme [EC 2.4.1.25]</i>	33
2.2.3.3	<i>ADPglucose pyrophosphorylase [EC 2.7.7.27]</i>	33

2.2.3.4	<i>Soluble starch synthase [EC 2.4.1.21]</i>	34
2.2.3.5	<i>Starch phosphorylase [EC 2.4.1.1]</i>	34
2.2.3.6	<i>α-amylase [EC 3.2.1.1]</i>	34
2.2.3.7	<i>β-amylase [EC 3.2.1.2]</i>	35
2.2.3.8	<i>β-glucuronidase</i>	35
2.2.4	Sugar assays	36
2.2.4.1	<i>Preparation of sugar-containing extracts</i>	36
2.2.4.2	<i>Glucose</i>	36
2.2.4.3	<i>Fructose</i>	36
2.2.4.4	<i>Sucrose</i>	36
2.2.5	Starch	37
2.2.6	Amylose	37
2.2.7	Malto-oligosaccharide content	38
2.2.8	Iodine staining of intact leaves	38
2.2.9	[U- ¹⁴ C]sucrose metabolism in developing and mature tubers	38
2.2.9.1	<i>Tuber material</i>	38
2.2.9.2	<i>Supply of label</i>	38
2.2.9.3	<i>Incorporation of ¹⁴C into starch</i>	39
2.3	Physical measurements	40
2.3.1	Effect of irradiance level on plant growth	40
2.3.2	Grafting	40
2.3.3	Measurement of dry weight	41
2.4	Molecular procedures	41

2.4.1	Small scale extraction of plasmid DNA	41
2.4.2	Large scale extraction of plasmid DNA	41
2.4.3	Restriction enzyme digests	42
2.4.4	Electrophoresis	42
2.4.5	Ethanol precipitation	42
2.4.6	Dephosphorylation of DNA	43
2.4.7	Extraction of DNA from agarose gels	43
2.4.8	<i>E. coli</i> electro-competent cell production	43
2.4.9	Electroporation into <i>E. coli</i>	44
2.4.10	Ligations	44
2.4.11	PCR amplification of D-enzyme cDNA containing novel restriction enzyme recognition sites	44
2.4.12	PCR analysis of <i>E. coli</i> DNA	44
2.4.13	<i>Agrobacterium tumefaciens</i> LBA4404 competent cell production	45
2.4.14	Electroporation of <i>A. tumefaciens</i>	45
2.4.15	Isolation of plasmid DNA from <i>A. tumefaciens</i>	46
2.4.16	Crude extraction of genomic DNA from plant leaves	46
2.5	Tissue culture protocols	46
2.5.1	Propagation of stock plants	46
2.5.2	Co-cultivation of leaf discs	47
2.5.3	Subsequent cultivation and regeneration	47
2.5.4	Microtuber production	47
2.6	SDS-Page electrophoresis	48

2.6.1	Extraction of protein for SDS polyacrylamide gel electrophoresis	48
2.6.2	Protein determination	48
2.6.3	Gel preparation	48
2.6.4	Semi-dry electroblotting of proteins onto nitrocellulose membrane	49
2.6.5	Immunodetection of protein on the membrane	49
3	Chapter 3. Biochemical and Growth Analysis of <i>Solanum tuberosum</i> L. cv. May Queen with Lowered D-enzyme Activity	51
3.1	Introduction	51
3.2	Results	54
3.2.1	Validation and optimisation of the D-enzyme activity assay	54
3.2.2	The effect of freezing and the extraction process on enzyme activity	55
3.2.3	Activities of starch metabolic enzymes in potato tubers with lowered D-enzyme activity	57
3.2.4	Flower colour	57
3.2.5	Tuber yields from four different harvests of potato plants with lowered D-enzyme activity	57
3.2.6	The growth of potato plants with lowered D-enzyme activity grown under low and high irradiance	60
3.2.7	Tuber yields of plants with lowered D-enzyme activity grown under low and high irradiances	60
3.2.8	Starch and amylose content of potato tubers with lowered D-enzyme activity	64
3.2.9	The effect on tuber yield of organ specific expression of D-enzyme activity achieved by grafting	64
3.2.10	Accumulation of sugars in tubers with lowered D-enzyme activity stored at 12 °C	69
3.3	Discussion	72

4 Chapter 4 Analysis of the Function of D-enzyme in Leaf and Tuber Starch Metabolism of <i>Solanum tuberosum</i> L. cv. May Queen	76
4.1 Introduction	76
4.2 Results	78
4.2.1 The effect of lowered D-enzyme activity on the malto-oligosaccharide content of leaves during darkness and light	79
4.2.2 The effect of lowered D-enzyme activity on starch breakdown during continuous darkness	79
4.2.3 The effect of lowered D-enzyme activity on leaf starch breakdown during continuous light	80
4.2.4 The effect of lowered D-enzyme activity on the starch content of dark and light grown plants as detected by iodine staining	80
4.2.5 The effect of lowered D-enzyme on the amylose content of leaf starch	80
4.2.6 Validation of the [U ¹⁴ C]sucrose labelling experiment	84
4.2.7 The incorporation of [U ¹⁴ C]sucrose into starch in tubers with lowered D-enzyme activity	86
4.3 Discussion	89
5 The Production of Transgenic <i>Solanum tuberosum</i> L. cv. Prairie Plants with Altered D-enzyme Activity	94
5.1 Introduction	94
5.2 Results	96
5.2.1 Construction of plant transformation plasmids	96
5.2.2 Characterisation of plasmid constructs	96
5.2.2.1 Restriction endonuclease digest and PCR analysis of plasmid DNA from electroporated <i>E. coli</i>	96
5.2.2.2 Sequence analysis of plant transformation construct	96

5.2.2.3	<i>Restriction endonuclease digest analysis of DNA from electroporated A. tumefaciens</i>	97
5.2.3	Production of transgenic plants	97
5.2.4	Protein screening of regenerated plants	102
5.2.5	D-enzyme activity screen	102
5.3	Discussion	107
6	Chapter 6. Analysis of <i>Solanum tuberosum</i> L. cv. Prairie with Lowered D-enzyme Activity	109
6.1	Introduction	109
6.2	Results	111
6.2.1	Activities of starch metabolic enzymes in Prairie and Prairie <i>glgC-16</i> tubers with lowered D-enzyme activity	111
6.2.2	Growth of Prairie and Prairie <i>glgC-16</i> plants with lowered D-enzyme activity	111
6.2.3	Tuber yield of Prairie and Prairie <i>glgC-16</i> plants with lowered D-enzyme activity	111
6.2.4	Tuber starch content and proportion of amylose in Prairie and Prairie <i>glgC-16</i> plants with lowered D-enzyme activity	114
6.2.5	Sprouting characteristics of Prairie and Prairie <i>glgC-16</i> tubers with lowered D-enzyme activity	114
6.2.6	The effect of lowered D-enzyme activity on the malto-oligosaccharide content of leaves from Prairie plants during light and darkness	117
6.2.7	The effect of lowered D-enzyme activity on leaf starch degradation in Prairie plants during darkness	122
6.2.8	The effect of lowered D-enzyme activity on leaf starch synthesis in Prairie plants during continuous light	122
6.2.9	The incorporation of [^{14}C]sucrose into Prairie and Prairie <i>glgC-16</i> tubers with lowered D-enzyme activity	122

6.3 Discussion	128
7 General Discussion	132
7.1 The effect of lowered D-enzyme activity on tuber starch metabolism	132
7.2 The effect of lowered D-enzyme activity on leaf starch metabolism	134
7.3 The influence of D-enzyme in leaves on the metabolism of tubers	135
7.4 Future work	136
8 Appendix	138
9 Bibliography	144

Chapter 1. Introduction

1.1 The potato

The genus *Solanum* includes over 2000 species; most are found in tropical and subtropical regions and less than a tenth are tuber-bearing (Burton, 1989). *Solanum tuberosum* L. is one of seven species of cultivated potato (Miller and Lipschutz, 1984). Potatoes are herbaceous, tetraploid plants which produce aerial shoots which die back at the end of each season. The plant perennates by means of underground tubers which develop from sub-apical regions of underground shoots known as stolons. Up to 80 % of potato tuber dry weight is comprised of starch (International Starch Institute, 1998). *Solanum tuberosum* L. was originally from the Andes but now is grown extensively and has become the world's fourth largest arable food crop. Annual production in 1997 was over 300 million tonnes. 7 million tonnes of this was grown in the UK (FAO Yearbook, 1998). As well as being an important food source starch extracted from potato tubers has various other food and non-food applications (section 1.2.1).

A main aim amongst potato breeders has been to increase tuber yield whilst maintaining or improving quality. Until recently this has been approached through traditional breeding methods. Now genetic manipulation is a tool which can be used towards this objective. In addition, engineering tubers to produce starch designed for specific purposes may soon be a possibility.

1.2 Starch

1.2.1 Occurrence and uses

Starch, the main carbohydrate reserve in higher plants, is highly evolved as an insoluble but readily biodegradable storage compound. Starch is present in virtually every type of tissue (Jenner, 1982). Starch comprises the main source of energy in the human and animal diet. In the UK 32 % of the average diet on a dry weight basis consists of starch (Gaillard, 1987). The largest proportion of this starch comes from cereals. 10^9 tonnes of starch is produced per annum

world-wide from cereal crops (Gaillard, 1987). Starch also has industrial uses in either a chemically modified or native form in products such as cosmetics, glue, paper and paint (International Starch Institute, 1998). Most of this starch is isolated from maize but cassava, potato and wheat are also important sources. Potato starch is produced mainly in countries of the European Union. Genetically modified starch has many potential uses including biodegradable starch-polyethylene films (Roeper, 1990) and osmotic dehydrating agents (Isse, 1995).

1.2.2 Components of starch

Starch is comprised of two α -glucan polymers, amylose and amylopectin, which make up approximately 20 % and 80 % of starch respectively (Martin and Smith, 1995). Both polymers are joined by $\alpha(1, 4)$ links and contain $\alpha(1, 6)$ linked branches. Amylose has a molecular weight of between 5×10^5 to 10^6 and contains infrequent branches at asymmetric intervals. In contrast, amylopectin has a molecular weight of between 10^7 to 10^9 and is highly branched with clusters of glucose chains occurring at regular intervals (for review see Manners, 1989; Takeda, 1984, 1986). For both polymers the degree of polymerisation, branch length and branching interval vary with botanical source (Takeda, 1984, 1986). Amylopectin contains small amounts of covalently bound monoester-phosphates at the C-6 and C-3 positions of glucose residues (Hizukuri *et al.*, 1970; Takeda and Hizukuri, 1982). Protein and small amounts of lipid, minerals and vitamins are also present in starch granules (Burton, 1989).

1.2.3 Starch structure

Starch occurs as semi-crystalline granules which exhibit several levels of organisation (figure 1). At the lowest level of organisation granules are comprised of alternating crystalline and amorphous rings with a periodicity of 120-400 nm (French, 1984). These crystalline areas are thought to be formed by the specific structure of amylopectin within these regions. Amylopectin molecules contain regularly spaced clusters of chains of 12-20 glucose units. These are joined by longer chains of around 40 glucose units which span at least two of the clusters. It is this distinctive polymodal distribution of chain lengths which is thought to enable amylopectin to become organised into a semi-crystalline granule. It is widely accepted that the short chains

within clusters form double helices, not always straight or of uniform width, and these pack in ordered arrays to form crystalline lamellae (French, 1984). Granule crystallinity has been characterised into three main X-ray diffraction patterns, A, B or C depending on the packing patterns of amylopectin double helices in the crystalline lamellae. Studies employing advanced electron microscopy and neutron scatter have led to proposals that the semi-crystalline regions are organised into interconnecting suprahelical structures (Oostergetel and van Bruggel, 1993) and into discrete 'blocket' domains (Gallant *et al.*, 1997). Recently a 2 μm -wide X-ray beam was used to scan across single granules to analyse the structure (Waigh *et al.*, 1996, 1997, 1998). It was found that amylopectin double helices are arranged radially so that the crystals are tangential to the granule surface. This radial arrangement of chains suggests that monomer units are added to growing ends sticking out of the granule surface. This indicates that the side-chain liquid crystal polymer properties of amylopectin branch chains are sufficient for the formation of double helices and the crystallisation of the granule. This model is consistent with, but does not confirm, the suprahelical and 'blocket' models and provides a less complex explanation for starch granule assembly.

Amorphous areas in starch granules occur both as distinct rings and also as alternating rings within the semi-crystalline regions of the granule. They are thought to contain amylose and long chain sections from amylopectin (Jane *et al.*, 1992). The simultaneous synthesis of amylose and amylopectin and the determination of branch lengths in amylopectin are thought to be the main factors which need to be elucidated before the mechanism of starch granule synthesis is fully understood.

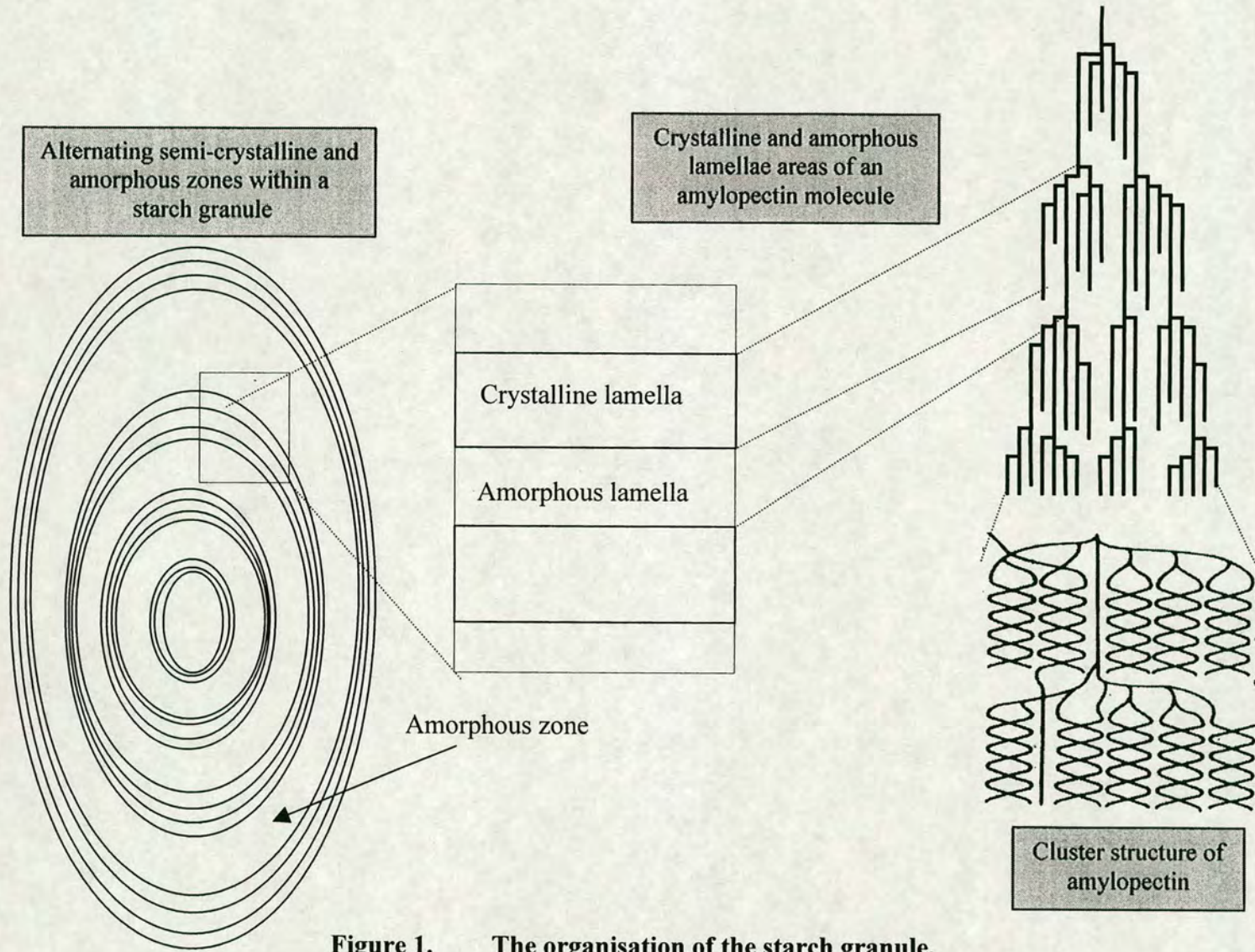


Figure 1. The organisation of the starch granule.

1.2.4 Transitory and storage starch

Transitory starch is formed within chloroplasts during photosynthesis and is degraded in the following dark period for respiration and growth. Storage starch accumulates in storage organs such as seeds, fruits and tubers. It is stored over longer periods and is remobilised in response to developmental signals.

The pattern of leaf starch accumulation corresponds strongly to the diurnal cycle. In light, when CO₂ fixation exceeds demand for photoassimilates, the excess is deposited as starch. In darkness transitory starch is mobilised from leaves to supply carbon to other parts of the plant. Leaf starch granules are usually smaller (Badenhuizen, 1969) and may have less crystalline order than storage starch granules (Radwan and Stocking, 1957). In spinach leaves starch granules contain a crystalline core and outer amorphous region which is more readily degraded in darkness (Steup *et al.*, 1983; Beck, 1985). Generally, transitory starch contains amylopectin molecules with shorter chain length branches and lower molecular weight amylose than that of storage starch (Matheson, 1996; Tomlinson *et al.*, 1997; Hizukuri, 1986). In non-photosynthetic tissues such as potato tubers, starch is synthesised and stored in specialised plastids called amyloplasts. Amyloplasts are heterotrophic, *i.e.* the import of carbon and energy precursors over the plastid membrane is required to support starch synthesis (Tjaden *et al.*, 1998). Storage starch is a long term energy supply controlled by genetic and developmental signals (Emes and Neuhaus, 1997). In cauliflower amyloplasts and potato tubers pulse-chase labelling experiments have shown that starch breakdown may occur during net starch synthesis, indicating that storage starch is not necessarily a stable end product in these plants (Neuhaus *et al.*, 1995; Sweetlove *et al.*, 1996b).

1.3 Starch Synthesis

1.3.1 Overview

In plants, photosynthetically derived carbon is translocated through the phloem mainly in the form of sucrose. Sucrose is thought to move into the cytosol of net-carbon importing parenchyma cells via plasmodesmata (ap Rees and Morrell, 1990). In the cytosol, sucrose is

likely to be glucosyltransferased by sucrose synthase to form UDPglucose and fructose which are further metabolised into hexose phosphates (Morrell and ap Rees, *et al.*, 1986; Zrenner *et al.*, 1995). Hexose phosphates have been shown to enter amyloplasts *in vitro* as precursors for starch synthesis (e.g. Neuhaus *et al.*, 1993; Wischmann *et al.*, 1999); however, the exact form of the imported hexose phosphates is unclear. In maize and barley endosperm isoforms of ADPglucose pyrophosphorylase (AGPase) have been located to the cytosol (Thorbjornsen *et al.*, 1996; Denyer *et al.*, 1996a). In maize endosperm a putative ADPglucose transport protein has been identified in the plastid membrane (Cao *et al.*, 1995; Sullivan and Kaneko, 1995). These data have led to the suggestion that in some species ADPglucose may be produced in the cytosol and transported into the plastid for starch synthesis (Pozueta-Romero *et al.*, 1991; Mohlmann *et al.*, 1997). For the initiation of starch synthesis a primer is required (Moreno, 1986). Several candidates have been proposed; however, the nature of initiation remains largely unresolved. UDP-glucose:protein transglucosylase, a UDP-glucose dependent self-glycosylating protein has been shown to initiate protein bound α -glucan synthesis *in vitro* (Bocca *et al.*, 1997). Alternatively, starch phosphorylase has been reported to produce primers which can be used by starch synthase (Baxter and Duffus, 1973).

ADPglucose pyrophosphorylase [EC 2.7.7.27, AGPase] catalyses the first step in starch synthesis by producing ADPglucose from glucose-1-phosphate and ATP. **Starch synthase** [EC 2.4.1.21, SS] catalyses the transfer of the glucosyl group from ADPglucose to the non-reducing end of an α -1,4-glucan chain (Priess, 1991). **Starch branching enzyme** [EC 2.4.1.18, SBE] forms branch points by catalysing the cleavage of α -1,4-glucosidic bonds and transferring the α -glucans released by formation of α -1,6 linkages. The mechanism by which phosphate residues are incorporated into starch is unclear. It has been demonstrated that incorporation of phosphate occurs concurrently with starch synthesis (Nielsen *et al.*, 1994). Recently a protein, R1, was identified in potato tubers which was reported to influence the phosphorylation status of starch (Lorbeth *et al.*, 1998).

1.3.2 Regulation of starch synthesis

AGPase is thought to catalyse a regulatory step in starch synthesis (Smith *et al.*, 1997). AGPase in plants is allosterically activated by 3-phosphoglyceric acid (3-PGA) and inhibited by inorganic phosphate (Pi) (Priess, 1988). There is good correlative evidence to show that changes in 3-PGA and Pi are involved in the regulation of starch synthesis in leaves (Stitt, 1997). This enables starch synthesis to be co-ordinated with carbon assimilation and sucrose synthesis. In addition, AGPase also exerts substantial influence on the rate of starch synthesis in leaves with a flux control coefficient of up to 0.6 (Neuhaus and Stitt, 1990). The regulation of starch synthesis in non-photosynthetic tissue is not fully understood. Recently it has been shown that AGPase exerts considerable control over starch synthesis in potato tubers (Sweetlove *et al.*, 1999). This could enable starch synthesis to be co-ordinated with rates of glycolysis so that the use of carbon for processes other than starch synthesis can be regulated. However, environmental factors such as severe water stress have been reported to lower the degree of control of AGPase on starch synthesis in potato tubers (Geigenberger *et al.*, 1999a). Regulation of AGPase by factors other than 3-PGA and Pi has also been suggested to occur in potato tubers (Geigenberger *et al.*, 1994). A plastidial phosphodiesterase which catalyses the hydrolysis of ADPglucose has been reported in several cereals and dicotyledonous species. The authors suggested the enzyme could affect starch synthesis by controlling levels of ADPglucose (Rodriguez-Lopez *et al.*, 2000).

1.3.3 Mechanism of starch granule synthesis

The interlinking between genetics, starch structure and enzyme function is poorly understood in plant metabolism. The activities of AGPase, SS and SBE are sufficient to account for all the types of linkages found in starch; however, the synthesis of a starch granule is more complex than can be explained by their catalytic properties alone. In addition, starch synthetic enzymes exist as multiple isoforms with spatial and temporal patterns of expression.

1.3.3.1 Amylose

Studies from mutant plants have shown that in storage organs one isoform of SS, granule bound starch synthase I (GBSS I), which is exclusively bound to starch granules, is entirely responsible

for amylose synthesis. For example, *waxy* mutants of maize and barley endosperm (Weatherwax, 1922; Rohde *et al.*, 1988), the *amf* mutation of potato (Hovenkamp-Hermelink *et al.*, 1987), and the *lam* mutation in pea embryos (Denyer *et al.*, 1996b) result in a complete lack or severe reduction of GBSS I and the production of amylose free starch. Multiple isoforms of GBSS I have been identified in pea leaves and wheat pericarp (Tomlinson *et al.*, 1998; Nakamura *et al.*, 1998). In potato one isoform of GBSS I is responsible for amylose synthesis in both leaves and tubers (Jacobsen *et al.*, 1989). GBSS I may also be involved in amylopectin synthesis. GBSS I has been reported to elongate amylopectin chains in isolated starch granules (Denyer *et al.*, 1996b). In addition, mutant plants which are deficient in GBSS I have been reported to exhibit altered amylopectin structure (e.g. Takeda and Hizukuri 1987; Flipse *et al.*, 1996a). The mode of action of GBSS I has been studied extensively *in vitro*. When isolated starch granules containing GBSS I were supplied with ADPglucose and malto-oligosaccharides (MOS), MOS were processively elongated into long glucan chains within the granule matrix. In the absence of MOS amylopectin chains were elongated (Denyer *et al.*, 1996b). It was also found that soluble amylopectin was required for the elongation of MOS by GBSS I suggesting a specific interaction between GBSS I and amylopectin may determine the activity of GBSS I (Denyer *et al.*, 1999). Under conditions where ADPglucose supply is limiting, amylose:amylopectin ratios are frequently lowered (e.g. Lloyd *et al.*, 1996; Tjaden *et al.*, 1998). This may be due to different affinities of GBSS I and soluble starch synthase (SSS) for ADPglucose (Clarke *et al.*, 1999). In starch granules from *Chlamydomonas reinhardtii*, GBSS I can also synthesise amylose by elongating amylopectin chains which are then cleaved by an unknown mechanism (van der Wal. *et al.*, 1998). There is no evidence of this occurring in higher plants.

The distribution of amylose within starch granules is not uniform. In potato tubers, for example, amylose is more concentrated at the periphery of the granule; the molecular weight of amylose at the centre of the granule is greater than at the periphery and the amylose content increases with granule size (Jane and Shen, 1993). GBSS I is thought to become embedded within the granule and synthesise amylose from within the semi-crystalline matrix rather than at the

granule surface (Tatge *et al.*, 1999). This supports the idea that the space available inside the granule may be an important determinant of amylose synthesis (Flipse *et al.*, 1996a). The exact association of amylose with amylopectin in starch granules is highly complex and remains to be clearly defined.

1.3.3.2 Amylopectin

The mechanism by which the polymodal distribution of amylopectin chain lengths arises is thought to be of critical importance in determining the structure, and hence properties, of intact starch granules. The actions of multiple isoforms of SS and SBE may play an integral part in determining the structure of amylopectin. Soluble starch synthases (SSS) can be divided into three classes SS I, SS II and SS III, based on their amino acid sequence. The occurrence of particular isoforms varies between species, tissue and developmental stage (e.g. Abel *et al.*, 1996; Li *et al.*, 1999). All three classes are found in potato tubers (Edwards *et al.*, 1995; Abel *et al.*, 1996; Marshall *et al.*, 1996). Mutants and transgenic plants lacking specific isoforms of SSS have been used to study the role(s) of these isoforms. Based on this work it is thought that individual isoforms play specific roles in amylopectin synthesis. For example, the *rug5* mutation in pea eliminates SS II and results in amylopectin with fewer intermediate and more short and long length chains. This implies that SS II may have a role in producing intermediate length amylopectin chains, a function which cannot be compensated for by other isoforms (Craig *et al.*, 1998). The *dull 1* mutation in maize results in a deficiency in a SSS similar to SS III of potato. In the starch from these plants amylopectin was found to exhibit an increased branching frequency and amylose content was increased. However, in *dull 1* the occurrence of pleiotropic effects on other enzymes has made it difficult to ascribe a precise role for this enzyme (Gao *et al.*, 1998). In potato, lowering SS I or SS II through antisense technology was not found to result in any major changes to the amount or structure of starch at a gross level (Edwards *et al.*, 1995; Kossmann *et al.*, 1997; for review see Smith *et al.*, 1997); whereas reduction of SS III was reported to result in starch with altered granule morphology, indicating a change to amylopectin structure (Abel *et al.*, 1996). When SS II and SS III were lowered through antisense technology in the same potato tubers the resulting starch exhibited characteristics which were not predicted

from lowering each of the isoforms individually (Edwards *et al.*, 1999; Lloyd *et al.*, 1999). Severe changes occurred to granule morphology, amylopectin chain lengths and gelatinisation behaviour. These data imply that SSS isoforms act synergistically during starch synthesis. The specific action of a particular isoform is likely to depend on factors such as substrate structure and genetic, environmental and developmental background as well as intrinsic catalytic properties (Edwards *et al.*, 1999).

Starch branching enzymes form branch points by catalysing the cleavage of α -1,4-glucosidic bonds and transferring the α -glucans released by formation of α -1,6 linkages. Like the starch synthases, starch branching enzymes are also divided into two classes, A and B, based on amino acid sequence (Burton *et al.*, 1995). As with SSS, expression of SBE isoforms varies according to species, tissue and developmental stage. For example, potato tubers contain both classes whereas potato leaves contain only the A class (Larsson *et al.*, 1998). The A and B isoforms of maize endosperm studied *in vitro* and expressed in *E. coli* exhibited differences in the type of chains they transferred. A classes preferentially transferred shorter chains and had a lower affinity for amylose than B classes (Takeda *et al.*, 1993; Guan *et al.*, 1995). It is thought these characteristics may follow for all members of the A and B classes (Smith *et al.*, 1999). In pea embryos the expression of SBE isoforms was reported to correlate with changes in amylopectin chain length (Burton *et al.*, 1995). From these results it has been suggested that different isoforms of SBE have specific roles in amylopectin synthesis (Priess and Sivak, 1996). Antisense inhibition of SBE A in potato was found to result in starch which contained an increase in the average chain length in amylopectin and a significantly branched 'intermediate' material whereas amylose content was unchanged (Jobling, *et al.*, 1999). This is despite SBE A comprising only 2 % of wild type SBE activity in potato tubers (Jobling, *et al.*, 1999). This phenotype was similar to SBE A mutants of pea (*r*) and maize (*ae*), although in these plants amylose contents were also increased (Tomlinson *et al.*, 1997; Shi *et al.*, 1998). It is important to mention that the occurrence of pleiotropic effects in some of these plant lines has hindered subsequent experimental interpretation (Tomlinson *et al.*, 1997). The polymodal chain distribution of amylopectin was not affected in pea plants lacking SBE A (Lloyd *et al.*, 1996;

Tomlinson *et al.*, 1997). This is in contrast to the SSS mutants *rug5* and *dull1* described previously which do exhibit altered chain length distribution. This indicates that SSS may be more important than SBE in determining the polymodal branch chain length. A reduction in the expression of SBE B through antisense technology was found to have little effect on potato tuber starch (Safford *et al.*, 1998) but inhibition of both SBE A and B in potato resulted in high amylose starch which lacked an amylopectin component (Schwall *et al.*, 2000). These results indicate that SBE isoforms may act synergistically, as lowering the activity of both isoforms resulted in a phenotype which was not predicted from lowering each isoform individually.

There is substantial evidence to suggest that debranching enzymes (DBE) are also involved in starch synthesis. DBE is known to be involved in starch degradation in germinating or sprouting storage organs (Manners, 1985), but it is also reported to accumulate in organs which actively synthesise starch such as maize kernels and pea embryos (e.g. Doeblert and Knutson, 1991; Sun *et al.*, 1999). DBE catalyse the hydrolysis of α -1,6-linkages and fall into two classes: pullulanases hydrolyse α -1,6-linkages in the yeast polysaccharide pullulan (maltotriose units linked end to end by α -1,6-linkages) and limit-dextrins of amylopectin (Nakamura, 1996). Isoamylases cannot attack pullulan but can debranch amylopectin and its limit-dextrins (Doeblert and Knutson, 1991). Plastidial and extraplastidial forms of DBE have been reported in several species (e.g. Li *et al.*, 1992; Zhu *et al.*, 1998). Mutations which eliminate DBE in maize endosperm (*sugary1*), rice endosperm (*sugary1*) and *A. thaliana* (*dbel*) result in the accumulation of a highly branched soluble glucan called phytoglycogen, as well as a reduction in starch content (James *et al.*, 1995; Nakamura *et al.*, 1997; Zeeman *et al.*, 1998). The *sta7* mutant from *C. reinhardtii* is deficient in DBE activity and is reported to produce phytoglycogen instead of starch (Mouille *et al.*, 1996). The phenotypes of these mutants suggest that DBE is required for normal starch synthesis but the exact role of DBE in starch synthesis is unknown.

1.3.4 Models of amylopectin synthesis

Pre-amylopectin trimming model. The phenotypes of mutant plants which lack DBE activity led to the proposal of a model for amylopectin synthesis in which DBE plays an integral role

(Ball *et al.*, 1996). In this model it is suggested that SSS and SBE elongate clusters of branches at the surface of the granule to produce a highly branched, disorganised glucan termed pre-amylopectin. Debranching enzyme is then required to selectively trim pre-amylopectin to a form which can be further elongated by SSS and SBE.

Although this model provides an explanation for the synthesis of the amylopectin cluster structure and the periodicity of semi-crystalline layers there is no direct evidence suggesting that it occurs *in vivo*. The enzymes responsible for the accumulation of phytoglycogen in DBE mutants of cereal or *C. reinhardtii* have not been specifically identified. For example, in the *sugary1* mutant of maize the mutation is known to occur in a gene encoding isoamylase (James *et al.*, 1995). However, there are also secondary effects on pullulanase (Rahman *et al.*, 1998) and soluble starch synthase (Singletarry *et al.*, 1997) activities in these plants. In rice endosperm *sugary1* has been shown to be encoded by an isoamylase gene and it has been reported that pullulanase and isoamylase are responsible for amylopectin synthesis in this tissue (Kubo *et al.*, 1999).

Soluble glucan model. Based on the phenotype of *dbe1* plants it was suggested that DBE is not directly involved in amylopectin synthesis, but along with other starch metabolising enzymes it plays a role in recycling soluble glucans produced by the action of SSS and SBE on malto-oligosaccharides (MOS) present in the stroma (Zeeman *et al.*, 1998a). Although SS and DBE are thought to elongate the surface of the granule they can also metabolise small MOS which are likely to be present in the plastid stroma. In the absence of DBE, MOS will be elongated by SS and SBE into soluble glucans which would result in phytoglycogen accumulation.

The *dbe1* mutant of *A. thaliana* lacks an isoamylase but no other effects on enzymes of starch metabolism were detected in these plants (Zeeman *et al.*, 1998a). During the day *dbe1* plants were found to accumulate starch and a highly branched soluble glucan similar to the phytoglycogen of *sugary1* mutants. The starch which does accumulate in *dbe1* was found to be

identical to starch from wild type plants. Amylopectin and phytoglycogen occur together in the same chloroplasts in *dbe1*. These observations are not consistent with the pre-amylopectin trimming model. According to this model loss of debranching activity would result in the formation of a single type of glucan with a structure intermediate between amylopectin and phytoglycogen. Alternatively, if other debranching activity could compensate for the loss of isoamylase then only starch would be expected to accumulate. Although the *sugary1* mutants of maize and rice do contain some starch it is not known whether this accumulates at the same time or in the same plastids as phytoglycogen. This model could also explain the presence of starch degradative enzymes during periods of starch accumulation (section 1.4).

There is no direct evidence showing that either the pre-amylopectin trimming model or the soluble glucan model occur *in vivo*.

1.4 Starch degradation

The biochemistry and spatial organisation of starch degradation is complex. Starch may be degraded by hydrolysis, phosphorolysis or both. Starch degradative enzymes exist as multiple isozymes: most of which appear to be cytosolic. As starch granules are only accessible to enzymes within the plastid, cytosolic enzymes are probably not directly involved in starch degradation. Less is known about the degradation of starch than is known about its synthesis. The information gained to date has mainly come from the identification and characterisation of starch-degrading enzymes. Some of the analysis undertaken on starch degradative enzymes has been prone to experimental problems. Ambiguities arising from possible contamination of chloroplast extracts and different specificities of biochemical assays may account for some of the apparent anomalies.

1.4.1 Enzymes of starch degradation

α -**amylase** [EC 3.2.1.1] (endoamylase) hydrolyses α 1,4-links in glucan polymers by transferring a glucosyl group to water (Preiss and Levi, 1980). Internal bonds of α -glucans are

cleaved randomly to yield products with a new reducing group. α 1,6-links and links adjacent to non-reducing ends are not hydrolysed. α -amylases are able to attack intact starch granules and therefore may have a role in the initiation of starch degradation (Steup, 1988). They may also produce oligosaccharides for further hydrolytic and phosphorolytic breakdown (Steup, 1983). Multiple isoforms of α -amylase have been reported in several species (Beck and Ziegler, 1989). Some of these have been identified as chloroplastic; for example, four chloroplastic isoforms have been isolated in sugar beet (Li *et al.*, 1992) and two in *A. thaliana* (Zeeman *et al.*, 1998b; Kakefuda *et al.*, 1997). An apoplastic location for α -amylase has also been reported (Li *et al.*, 1992). The activity of α -amylase is low or even undetectable in potato tubers (Davies and Ross, 1986; Morrell and ap Rees, 1986).

β -amylase [EC 3.2.1.2] (exoamylase) hydrolyses α 1,4-links in amylose and amylopectin by cleaving maltose from the non-reducing end of the chain. α 1,6-links are not hydrolysed. In *Vicia faba*, barley, wheat, soybean, sweet potato and pea most of the β -amylase activity is extraplastidial and possibly vacuolar (Ziegler and Beck, 1986; Beck and Ziegler, 1989). Two chloroplastic β -amylase isoforms have been identified in pea (Kakefuda *et al.*, 1986) and chloroplastic β -amylase was reported to be strongly expressed in the leaf palisade layer and vascular strands of petioles and stems in *A. thaliana* (Lao *et al.*, 1999). β -amylase has been reported to be present as a storage protein in alfalfa roots (Gana *et al.*, 1998). The activity of β -amylase has been reported to be greater than α -amylase in stored and sprouting potato tubers (Cottrell *et al.*, 1993; Davies and Ross, 1986).

α -glucosidase [EC 3.2.1.20] has wider substrate specificity than the amylases. It can hydrolyse α -D-glucosides, oligosaccharides and polysaccharides. Most α -glucosidases will cleave α 1,2-, α 1,3-, α 1,4- and α 1,6-linked glycosidic bonds. α -glucosidases have been reported to attack isolated native starch granules in barley kernels and pea chloroplasts (Sun and Henson, 1990; Sun *et al.*, 1995). Plastidic α -glucosidases have been identified in pea leaves (Sun *et al.*, 1995). Low activity of α -glucosidase has been detected in *A. thaliana* leaves (Critchley, 1999). α -

glucosidases have been characterised in starch storing organs including barley seeds (Sun and Henson, 1990) and potato tubers (Killilea and Clancy, 1978). The activity of α -glucosidase in potato tubers is low compared to amylases (Cottrell *et al.*, 1993).

Debranching enzyme hydrolyses α 1,6-links in α -glucan chains. Generally, debranching enzymes are assumed to have a role in starch degradation (Beck and Ziegler, 1989). Two classes have been identified, isoamylase and pullulanase. The exact role of each isoform is unclear. Debranching enzymes have also been linked to roles in starch synthesis (section 1.3.3.2). Chloroplastic debranching enzymes have been identified in several species including spinach, pea and *A. thaliana* (Okita *et al.*, 1979; Kakefuda *et al.*, 1986; Zeeman *et al.*, 1998a).

Starch phosphorylase [EC 2.4.1.1] catalyses the reversible production of glucose-1-phosphate (G-1P) from organic phosphate (Pi) and α -glucan. The concentration of *in vivo* substrates suggests a degradative role for the enzyme. The ratio of Pi:G-1P in chloroplasts of approximately 125 (Wirtz *et al.*, 1980) is at least 50-fold greater than the phosphorylase equilibrium constant at pH 7.3 (Cohn, 1961). Two types of starch phosphorylase, Pho 1 and Pho 2, have been identified in plant tissue. Pho 1 is plastidic and has low affinity for highly branched glucans (Steup and Latzko, 1979; Nakano and Fuki, 1986). Pho 2 is cytosolic and has higher affinity for branched glucans and a high-molecular weight heteroglycan (Conrads *et al.*, 1986; Shimomura *et al.*, 1982; Yang and Steup, 1990). Two Pho 1 isoforms, Pho 1a and Pho 1b, have been identified in potato. Pho 1a accumulates strongly in amyloplasts and weakly in chloroplasts. Pho 1b accumulates to an equal extent in both types of plastid (Sonnewald *et al.*, 1995). Phosphorylases probably act on soluble glucans produced by α -amylases (Stitt and Steup, 1985). Surprisingly, starch phosphorylase mRNA is co-expressed with genes encoding starch biosynthetic enzymes (St-Pierre and Brisson, 1995; Duwenig *et al.*, 1997b). Plastidic phosphorylase gene expression is reported to be induced by sugars (St-Pierre and Brisson, 1995; Duwenig *et al.*, 1997b). Starch phosphorylase protein is regulated at the post-transcriptional level (St-Pierre and Brisson, 1995).

Maltose phosphorylase [EC 2.4.1.8] catalyses the conversion of maltose and Pi to glucose and G-1P. The enzyme has been reported to be present in low quantities in pea (Levi and Priess, 1978). There are few other reports of its occurrence. The enzyme could potentially degrade the products of amylolytic action, although it has also been proposed to have a role in maltose synthesis (Kruger and ap Rees, 1983).

1.4.2 Starch degradation in photosynthetic organs

In chloroplasts, starch degradation has been found to respond to day length (Britz, 1990) and the rate of starch synthesis during the previous day (Lin *et al.*, 1988b). In addition, the rate of starch degradation in light has been reported to respond to the rate of photosynthesis (Fox and Geiger, 1984). These data indicate that the degradation of transitory starch is a regulated process. The specific enzymes and mechanisms involved have yet to be clearly defined. Isoforms of α -amylase, β -amylase, starch phosphorylase, D-enzyme and debranching enzyme have been located to chloroplasts in several species (Okita *et al.*, 1979; Lin *et al.*, 1988a; Kakefuda *et al.*, 1986). Surprisingly, many starch degrading enzymes are present constitutively in photosynthetic tissues.

Hydrolytic and phosphorolytic pathways are both believed to be involved in leaf starch degradation (Steup, 1983; Beck and Ziegler, 1989). In *A. thaliana* hydrolytic activity is reported to exceed phosphorolytic activity 18-fold (Lin *et al.*, 1988a) and, in addition, hexose transport through the chloroplast membrane was found to be required for maintenance of normal rates of starch degradation (Trethewey and ap Rees, 1994). A mutant of *A. thaliana*, *SZ63*, which is impaired in its ability to degrade starch, was found to be deficient in a chloroplastic α -amylase (Zeeman *et al.*, 1998b). This was the first description of a specific enzyme which is required for starch degradation in leaves. In the *A. thaliana* isoamylase mutant, *dbel*, cleavage of α 1,6-links during the night was not affected by the loss of isoamylase activity, indicating an alternative activity was responsible for this action (Zeeman *et al.*, 1998a). Correlative evidence indicates that β -amylase may be involved in transitory starch degradation. Maltose is the major product of amylopectin degradation in isolated pea chloroplasts (Kakafuda *et al.*, 1986) and β -amylase

activity is several fold higher than the activities of other starch degradative enzymes in leaves of *A. thaliana* (Critchley *et al.*, 2000). It has been suggested that starch is likely to be degraded by phosphorolysis in pea leaves (Stitt *et al.*, 1978). However, conflicting reports describe the occurrence of amylase activities in pea leaves. In one study, two isoforms of chloroplastic β -amylase were identified from pea leaves but α -amylase was not detected (Kakefuda *et al.*, 1986), whereas in another study α -amylase was detected (Ziegler and Beck, 1986). Potato leaves with chloroplastic Pho 1b activity lowered by antisense technology were found to exhibit normal leaf starch accumulation and it was suggested this may indicate the prevalence of amylolytic activity in potato leaves (Sonnewald *et al.*, 1995). A cytosolic polysaccharide fraction was isolated from spinach and pea leaves which was reported to interact strongly with Pho 2 (Steup *et al.*, 1991; Yang and Steup, 1990). The role of Pho 2 *in vivo* remains unknown

The regulation of individual degradative enzymes in leaves has been studied in some species. A dark-induced α -amylase from *A. thaliana* leaves has been identified which exhibits peak activity correlating with a decreasing leaf starch content (Kakefuda and Priess, 1997). In pea leaves, diurnal oscillations in chloroplastic pH were found to effect α -glucosidase activity and this was shown to correlate with patterns of starch degradation (Sun *et al.*, 1995). In *A. thaliana*, β -amylase mRNA was reported to accumulate in response to the addition of sugars and light (Mita *et al.*, 1995). In spinach and potato leaves, chloroplastic starch phosphorylase genes have been reported to be induced by sugars (Duwenig *et al.*, 1997b).

1.4.3 Starch degradation in storage tissues

In storage organs, starch is mobilised in response to developmental signals. In potato tubers this may be due to cold treatment, prolonged storage above 10 °C resulting in senescent sweetening or the onset of sprouting (Isherwood, 1973; Burton, 1989). Starch degradation which occurs during net starch synthesis is termed starch turnover and has been observed in both developing and mature potato tubers (Sweetlove *et al.*, 1996b). α -amylase, β -amylase, isoamylase, α -glucosidase, and starch phosphorylase activities have been detected in potato tubers (Kennedy and Isherwood, 1975; Killilea and Clancy, 1978; Cochrane *et al.*, 1991; Cottrell *et al.*,

1993). It is unclear whether storage starch degradation proceeds via a phosphorolytic or hydrolytic route. Evidence in support of both of these mechanisms is largely based on correlative data. Sowokinos, (1990) suggested that starch breakdown in potato tubers is primarily phosphorolytic. However, reports describing increases in phosphorolytic and amylolytic activities during starch mobilisation conflict. α -amylase and β -amylase have been reported to be induced by storage at low temperature (Cottrell *et al.*, 1993; Nielsen *et al.*, 1997). In contrast, Sowokinos *et al.* (1985) found no changes in amylase activity during cold-sweetening. In one study starch phosphorylase activity was reported to increase in response to cold (Claassen *et al.*, 1993) whereas Hill *et al.* (1996) found no effect on starch phosphorylase activity of cold treatment. These differences may be due to differences between cultivars or due to the fact that the assays employed did not differentiate between different enzyme isoforms. In potato plants with lowered expression of the R1 protein reductions in leaf and tuber, starch phosphorylation and starch degradability were observed (Lorbeth *et al.*, 1998). If the reduction in starch degradation was due to the change in starch phosphorylation this would suggest that the phosphorylation of starch is important for its degradability in potato.

The role of cytosolic isoforms of starch degradative enzymes are not understood. A reduction in the activity of cytosolic phosphorylase Pho 2, through antisense technology, was found not to affect starch quality or quantity (Duenwig *et al.*, 1997a). During sprouting, amyloplast membranes progressively disintegrate (Kumar and Knowles, 1993). This could enable cytosolic degradative enzymes to access starch granules (Ohad *et al.*, 1971).

1.4.4 Source-sink relations in potato

Plant organs which exhibit net import of carbohydrate are known as sinks. Organs from which the carbohydrate originates are known as sources (Willmitzer *et al.*, 1993). Source and sink status change during development and this results in competition and interactions over the fate of assimilated carbon. The flux of photosynthetically-derived carbon exported from leaf (source) tissue to developing potato tubers (sink) is a regulated process. The influence of leaf and tuber metabolism on this flux is the subject of a long-standing debate. Sink-source relations have been

widely studied at the physiological level (Wardlaw, 1990); more recently molecular techniques have been employed to investigate this topic.

It has been suggested that source metabolism is limited by the demand of sink tissue for carbon (Geiger, 1976). However, experiments designed to test this were inconclusive (Willmitzer *et al.*, 1993). In the long-term there is evidence to suggest that source activity is co-ordinated to sink demand through the effects of sucrose on photosynthetic genes. For example, in potato a reduction in sucrose export from leaves by expression of an apoplastic yeast derived invertase resulted in reductions in plant growth rate, number of tubers and a severe fall in photosynthetic capacity (Heineke *et al.*, 1992). The effect on photosynthetic capacity was shown not to be caused by a feedback regulation of sucrose synthesis but by changes to gene expression induced by sugar accumulation in leaves (Krapp *et al.*, 1993). Strong evidence suggests that the control of carbon flux through a potato plant is dominated by source tissue. For example, a reduction of cytosolic fructose-1,6-bisphosphatase activity in potato leaves was found to lead to a reduction in sucrose synthesis in light, but carbon export to tubers was unaffected due to increased starch breakdown and sucrose export in the night (Zrenner *et al.*, 1996). Measurements of flux control coefficients between source and sink 'blocks' in developing potato tubers found that up to 90 % of the control of flux was accounted for by source tissue (Sweetlove *et al.*, 1998; Sweetlove *et al.*, 2000). This finding may explain the failure of successive attempts to increase tuber yield by the manipulation of tuber metabolism (Muller-Rober *et al.*, 1992).

1.5 D-enzyme

1.5.1 Occurrence of D-enzyme

D-enzyme, disproportionating enzyme, or 4- α -glucanotransferase [EC 2.4.1.25] was first identified in potato tubers by Peat *et al.* (1956). It has since been found in leaves, stems, roots and stolons of potato (Takaha *et al.*, 1993). D-enzyme has also been found in tomato fruits (Manners and Rowe, 1969), carrot roots (Manners and Rowe, 1969), spinach leaves (Okita *et al.*, 1979), pea chloroplasts (Kakefuda *et al.*, 1986), germinated barley seeds (Yoshio *et al.*, 1986), sweet potato (Suganuma *et al.*, 1986), Norway spruce needles (Egger *et al.*, 1996) and *A.*

thaliana (Lin and Priess, 1988). In *A. thaliana*, D-enzyme was found in leaves, stems, stigmas, siliques, flower buds and petals (Critchley, 1999).

1.5.2 Reactions catalysed by D-enzyme

1.5.2.1 Disproportionation

The disproportionation reaction catalyses the cleavage of maltosyl or larger glucan moieties from 1,4- α -D-glucan donor molecules, followed by the transfer to 1,4- α -D-glucan acceptor molecules by reformation of the α -D-(1-4) link. The reaction is represented by the following equation:



This reaction has been well-characterised *in vitro* using chromatographic methods (Jones and Whelan, 1969; Takaha *et al.*, 1996). There are two 'forbidden' glucan linkages which cannot be cleaved by D-enzyme: the bond penultimate to the reducing end and the bond at the non-reducing end. Consequently, maltose does not function as a donor or acceptor molecule in reactions catalysed by D-enzyme. In addition, maltose is never produced by the action of D-enzyme. Maltotriose is the smallest possible donor molecule but glucans as large as amylose and amylopectin molecules can also act as donors. Glucose, glucans larger than maltose, amylose and amylopectin molecules can act as acceptor molecules. Long α -1,4-glucan units and highly branched cluster units of amylopectin can be transferred between molecules although maltosyl groups are transferred preferentially. Glucose is never transferred. Partly purified D-enzyme from *C. reinhardtii* has been found to catalyse glucan transfer within amylopectin molecules (Colleoni *et al.*, 1999a). The *in vivo* substrate for D-enzyme is unknown. A schematic representation of these reactions is shown in figure 2.

1.5.2.2 Cyclisation

Amylose and amylopectin can serve as donor and acceptor molecules for *in vitro* intramolecular transglycosylation reactions catalysed by D-enzyme, to produce cyclic $\alpha(1,4)$ -glucans

(cycloamylose) and cyclic $\alpha(1,4),\alpha(1,6)$ -glucans (Takaha *et al.*, 1996; Takaha *et al.*, 1998a). Cycloamylose has also been reported to be produced by the action of 4- α -glucanotransferase from *Thermococcus litoralis* (Jeon *et al.*, 1997). It is not known if cyclisation reactions occur *in vivo*. A schematic representation of these reactions is shown in figure 2.

1.5.3 Localisation and expression

D-enzyme activity has been found in both chloroplastic and cytosolic fractions of spinach and pea leaves (Okita *et al.*, 1979; Kakefuda *et al.*, 1986). In *A. thaliana* leaves, D-enzyme activity was found to be confined to the chloroplastic fraction (Lin *et al.*, 1988) and immuno-gold labelling indicated the presence of D-enzyme protein on starch granules (Critchley, 1999). D-enzyme cDNA from potato and *A. thaliana* encodes a putative transit peptide which is thought to target the protein to chloroplasts and amyloplasts (Takaha, 1996; Critchley, 1999).

In potato leaves D-enzyme mRNA accumulates in response to sucrose, glucose and light and is most abundant in developing and mature tubers (Takaha *et al.*, 1993). This suggests that D-enzyme gene expression increases during periods of starch synthesis. D-enzyme mRNA expression decreases in cold-stored and sprouting tubers, when starch is degraded but protein expression remains high (Takaha, 1996). In agreement, in *A. thaliana*, D-enzyme protein increases in tissue which actively synthesises starch and decreases in senescing tissue (Critchley, 1999). This expression pattern is similar to that of starch phosphorylase (section 1.4.1).

1.5.4 The function of D-enzyme

1.5.4.1 Mammalian glycogen metabolism

Higher non-plant Eukaryotes store α -1,4-D-glucan as glycogen rather than starch. Glycogen is chemically identical to starch but has a different molecular structure. Glycogen debranching enzyme (GDE) has two independent catalytic activities: α -1,4 \rightarrow α -1,4 glucanotransferase activity and α -1,6 glucosidase activity (debranching) (Yang *et al.*, 1992). GDE and glycogen phosphorylase are responsible for the complete degradation of glycogen. Glycogen

phosphorylase sequentially cleaves glucose-1-P from non-reducing ends of branches but cannot act on branches which are smaller than four residues. The glucanotransferase activity of GBE then catalyses maltotriosyl transfer from the shortened branches to a non-reducing end of the glycogen molecule, to enable further metabolism by glycogen phosphorylase. The remaining α -1,6-linked residue is removed by the glucosidase activity of GDE (for review see Takaha and Smith, 1999).

1.5.4.2 Microbial 4- α -glucanotransferases

Bacterial amylomaltases have up to 40.2 % amino acid sequence identity with potato D-enzyme (Takaha, 1996). The only difference between the enzymatic action of amylomaltase compared to D-enzyme is that it can transfer glucose between donor and acceptor molecules (Palmer *et al.*, 1976). The role of amylomaltase in *E. coli* is thought to involve disproportionating short malto-oligosaccharides (MOS) into longer chains which can be metabolised by glucan phosphorylase. Glucan phosphorylase cannot degrade MOS smaller than maltopentaose. In *E. coli*, amylomaltase is part of a MOS transport and utilisation system, which includes maltodextrin phosphorylase and maltose transport proteins (Schwartz, 1987). The genes for amylomaltase and glucan phosphorylase constitute the *malPQ* operon. In contrast, in *Haemophilus influenzae* and *Aquifex aeolicus*, the amylomaltase gene is on the glycogen operon, which also includes genes for

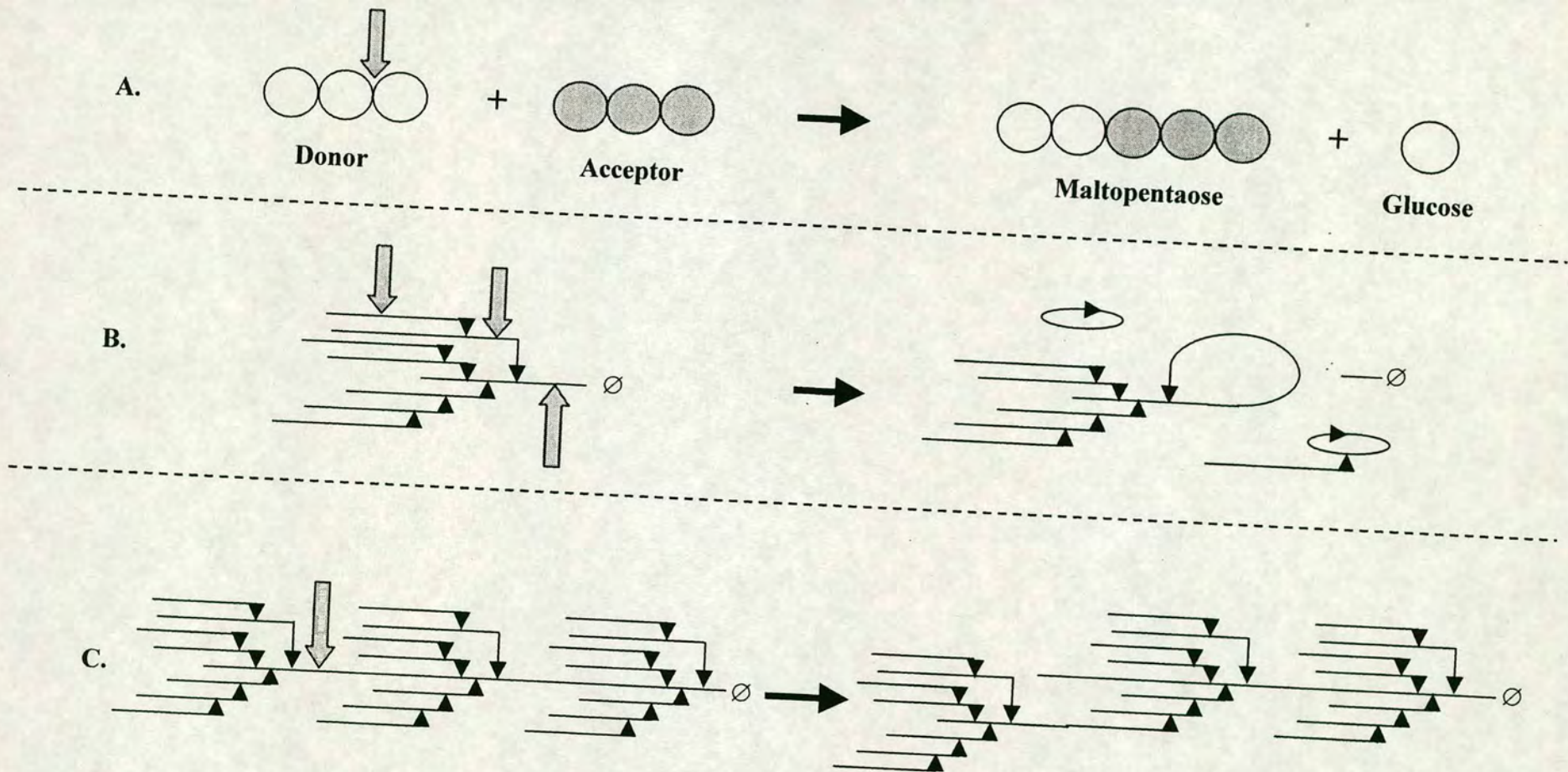


Figure 2. Schematic representation of reactions catalysed by D-enzyme *in vitro*.

A. disproportionation reaction between maltotriose donor and acceptor molecules resulting in the production of maltopentaose and glucose. B. Cyclisation reaction of an amylopectin molecule used both as a donor and acceptor. C. Disproportionation reaction within an amylopectin molecule. Linear or curved lines indicate α -1,4-glucan chains, vertical arrows indicate α -1,6-branch points. Grey-filled arrows triangles indicate the α -1,4-linkages which are attacked by D-enzyme. \emptyset indicates the glucosyl residue at reducing end.

glycogen synthesis and degradation (Fleischmann *et al.*, 1995; Deckart *et al.*, 1998). The absence of a MOS transport system in *H. influenzae* and *A. aeolicus* suggests that amylomaltase may be involved in glycogen metabolism in these organisms.

Cyclodextrin glucanotransferases (CGTases) are another type of 4- α -glucanotransferase which have been extensively studied in bacteria. By means of cyclisation reactions, CGTases convert starch into a mixture of cyclic α -1,4-glucans with degrees of polymerisation of 6, 7, or 8. CGTases can also catalyse disproportionation reactions (for review see, Kitahata, 1995). Some bacteria secrete amylase and CGTase to degrade glycogen or starch, possibly through disproportionation reactions as well as cyclisation reactions (for review see, Takaha and Smith, 1999). A cyclodextrin uptake system was reported in *Klebsiella oxytoca* (Fiedler *et al.*, 1996) indicating the potential importance of cyclodextrins in microbial α -D-glucan carbohydrate metabolism.

1.5.4.3 Role of D-enzyme in starch metabolism

The initial work undertaken on D-enzyme from plants involved extensive characterisation of its activity *in vitro* (section 1.5.2). From this it was proposed that D-enzyme is primarily involved in starch degradation (Lin and Priess, 1988; Kakefuda and Duke, 1989). It was suggested that D-enzyme may act to convert small MOS into larger MOS which can serve as substrates for starch phosphorylase. Starch phosphorylase cannot metabolise α -1,4-glucans smaller than maltopentaose (Steup and Schachtele, 1981). This system is similar to that described for amylomaltase (D-enzyme) from *E. coli* (section 1.5.4.2). Further *in vitro* work undertaken by Takaha (1996) led to the suggestion that cyclic glucans generated by D-enzyme could serve as substrates for starch degradative enzymes. Alternatively, cyclic glucans could be used to transport carbon through plants, a hypothesis which could explain the cytosolic location of many starch degradative enzymes (Beck and Ziegler, 1989) and the vascular location of β -amylase in *A. thaliana* (Lao *et al.*, 1999). A cyclic α -1,4-glucan transport system has been characterised in *K. oxytoca* (Fiedler *et al.*, 1996).

D-enzyme has also been proposed to have a role in starch synthesis. Takaha (1996) demonstrated that D-enzyme is able to transfer long α 1,4-glucan chains and branched α -glucans and that amylose and amylopectin can both act as acceptor molecules. From this it was suggested that D-enzyme could be involved in amylopectin synthesis by transferring α 1,4-glucans or cluster units between amylopectin or soluble pre-amylopectin molecules and in this way elongate the surface of the granule. Alternatively, D-enzyme and starch phosphorylase could be involved in metabolising MOS, released by the 'trimming' activity of debranching enzyme on amylopectin (Ball *et al.*, 1996) into glucose-1-phosphate which could be used by AGPase for starch synthesis. MOS generated by the action of debranching enzyme could also be metabolised by D-enzyme into substrates for starch synthase or branching enzyme. This could also explain the induction of D-enzyme and starch phosphorylase gene expression during starch synthesis (St. Pierre and Brisson, 1995; Takaha *et al.*, 1993). However, the views described above are speculative and are not supported with direct experimental evidence. Critically, all of this work was carried out *in vitro* and the *in vivo* substrate for D-enzyme is unknown.

Recent analysis of transgenic and mutant plants has enabled the effect of lack of D-enzyme on plant metabolism to be investigated. From this work two differing views have emerged as to the role of D-enzyme *in vivo*; one proposes that D-enzyme is involved in starch synthesis and degradation whereas the other model describes a role for D-enzyme in starch degradation.

A D-enzyme mutant isolated from *C. reinhardtii*, *stall-1*, was found to exhibit greater accumulation of MOS, lower starch content, higher amylose content in starch and a higher proportion of short chains in amylopectin compared to wild type (Colleoni *et al.*, 1999a). The authors argued this was due to a requirement for D-enzyme in normal starch synthesis. D-enzyme was shown to incorporate radiolabelled MOS into amylopectin *in vitro* and based on the pre-amylopectin trimming model (section 1.3.4). Colleoni *et al.* (1996b) suggested that D-enzyme disproportionates MOS released by the action of debranching enzyme into amylopectin at the granule surface. *Stall-1* also accumulated a water-soluble α -glucan polymer. According to the pre-amylopectin trimming model, phytyglycogen accumulates alongside starch when

starch synthesis is disrupted. The increase in proportion of amylose could result from a stimulation of amylose synthesis by MOS. MOS have been observed to act as primers for GBSS I activity in pea (Denyer *et al.*, 1996b). The presence of increased levels of MOS in *stall-1* is also consistent with a role for D-enzyme in facilitating the phosphorolytic degradation of starch through the metabolism of MOS.

C. reinhardtii is a unicellular, starch-storing alga which accumulates an α -glucan polymer similar to cereal endosperm starch when grown under nutrient starvation conditions. The phenotypes of *stall-1* described above were expressed to a maximum extent under nitrogen-starved conditions. It is unclear why starch content could be reduced by around 90 % in *stall-1* whilst other starch metabolic enzymes exhibited normal activities. This decreased starch phenotype was not observed under conditions in which *C. reinhardtii* synthesised transitory starch. No effects on growth were detected when *stall-1* was grown under varying light and growth conditions. Differences between the physiology and conditions required for starch accumulation suggest that direct comparisons between *C. reinhardtii* and higher plants should be made with caution (Manners, 1997).

In contrast to the work on *C. reinhardtii*, studies in higher plants have provided evidence that D-enzyme has a role in starch degradation. Potato plants with D-enzyme activity lowered by antisense technology to less than 3 % of wild type were produced and analysed by Takaha *et al.* (1998). Lack of D-enzyme resulted in slow plant growth and delayed sprouting of tubers, suggesting that D-enzyme may have a role in the mobilisation of starch for growth. The plants also exhibited a late flowering phenotype. Tuber starch content, proportion of amylose, molecular weight of debranched amylopectin and branch chain length were unchanged. It is possible that the level of D-enzyme activity in the tubers of these plants was sufficient for its normal *in vivo* role(s) to be fulfilled. Recently, a mutant of *A. thaliana*, *dpe1-1*, which contains a T-DNA insertion in the gene encoding D-enzyme, has been isolated and characterised. It was found that a dramatic accumulation of MOS occurred in *dpe1-1* in the dark. The MOS was comprised primarily of maltotriose (Critchley *et al.*, 2000). Plastidial starch phosphorylase

activity is low in *A. thaliana* chloroplasts and, as the enzyme is unable to metabolise α -glucans smaller than maltopentaose, the observed accumulation of maltotriose was likely to be the product of α -amylase and β -amylase activity (Zeeman *et al.*, 1998a; Lao *et al.*, 1999). This is strong evidence to suggest that D-enzyme has a role in starch breakdown by disproportionating small MOS into larger MOS which can be metabolised by starch degradative enzymes. It was also found that the rate of starch breakdown in darkness in *dpe1-1* plants was reduced compared to wild type. This could be due to a direct effect of the accumulation of MOS or it could be explained if D-enzyme has a direct role in degrading starch molecules. Under a normal diurnal cycle the amylose content of starch was higher in *dpe1-1*. This may have been an indirect consequence of a lack of D-enzyme as the high levels of MOS which accumulated during darkness could act as primers for GBSS I to synthesise amylose. MOS have been observed to act as primers for GBSS I activity in pea (Denyer *et al.*, 1996b). Rates of starch synthesis in *dpe1-1* were not affected by lack of D-enzyme. In addition, the chain length distribution of amylopectin was not altered. Therefore no evidence was found to support the proposal that D-enzyme has a role in the synthesis of amylopectin .

1.6 Project aims

Starch metabolism in photosynthetic and non-photosynthetic tissues has been widely studied. However, there are many deficiencies in our understanding of starch granule synthesis and more so in our knowledge of starch degradation. The isolation and analysis of *C. reinhardtii* and *A. thaliana* mutants which lack enzymes of starch metabolism has been integral in the development of new ideas concerning starch metabolism and, in particular, starch synthesis. A D-enzyme mutant from *C. reinhardtii* *stall-1* has provided indirect evidence that D-enzyme could have a role in starch synthesis in this alga. However, in plants D-enzyme has traditionally been linked to a role in starch degradation. The *A. thaliana* mutant *dpe1-1*, which contains a T-DNA insertion in the D-enzyme gene, has provided the first direct evidence for a specific role for D-enzyme. Based on the phenotypes of *dpe1-1*, strong evidence was found to suggest that D-enzyme is involved in the metabolism of MOS during starch degradation in darkness. In the present study knowledge gained from these recent advances was transferred to the study of D-enzyme in potato. The initial aims of this study were to characterise the phenotypes of potato plants from the cultivar May Queen with D-enzyme activity lowered to less than 3 % of wild type. Previous work on D-enzyme in potato has tended to focus on tuber metabolism. In this study the influence of D-enzyme activity in leaves on whole-plant metabolism was also investigated. The effect of lack of D-enzyme on tuber yield, leaf and tuber starch metabolism and leaf MOS content were analysed. Next, D-enzyme activity was lowered in a second cultivar of potato, Prairie. The effect of lowered D-enzyme activity in Prairie plants was compared to the effect of lack of D-enzyme in May Queen plants. D-enzyme activity was also lowered in Prairie plants which expressed the *glgC-16* gene from *E. coli*. Tubers from these plants exhibit exaggerated rates of starch synthesis and turnover. It was hypothesised that lowering D-enzyme activity in this background would result in large and detectable alterations to the starch metabolism of tubers from these plants.

Chapter Two. Materials and Methods

2.1 Materials

2.1.1 Plant material

Potato plants (*Solanum tuberosum* L.) cv. May Queen with D-enzyme activity lowered by antisense and co-suppression technologies were kindly donated by Dr Takeshi Takaha, Glico Co., Japan. The plants had been transformed using pBI101 based plasmids, pBIC102F and pBIC102R (Takaha *et al.*, 1998c). pBIC102F and pBIC102R contain homologous potato D-enzyme CND in sense or antisense orientation respectively, linked to a CaMV 35 S promoter. Controls were both wild type plants and plants which had been transformed with constructs containing a β -glucuronidase gene. Potato plants (*Solanum tuberosum* L.) cv. Prairie expressing the *glgC-16* gene from *Escherichia coli* were generously supplied by Advanced Technologies (Cambridge) Ltd, Cambridge, U.K.. These plants had been transformed using a pBIN19 (Bevan, 1984) based plasmid, pFW4173. pFW4173 contains a patatin promoter from genomic clones *ps3* and *ps27* (Blundy *et al.*, 1991) and a ribulose-1,5-bisphosphate carboxylase transit peptide from pea linked to the *glgC-16* gene (Ghosh *et al.*, 1992). In controls *glgC-16* was replaced by the β -glucuronidase gene. Levingtons F2 compost was from Fisons Ltd

Plants grown April-September were cultivated in pots of 25 cm diameter under natural light in an unheated greenhouse. Plants grown October-March were cultivated in pots of 20 cm diameter in a heated greenhouse at 20 °C, 16 h day length and at least 300 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ irradiance. Plants were grown in Levingtons F2 compost containing 30 % (v/v) grit.

2.1.2 Biochemical reagents and equipment

All chemicals and reagents were supplied by Sigma Chemical Co. Ltd., apart from EDTA and polyvinyl pyrrolidone, which were supplied by BDH Chemicals Ltd. and maltotriitol, which was from Wako Pure Chemical Industries Ltd. (Japan). Enzymes for use in assays were from Boehringer-Mannheim Corporation (Diagnostics and Biochemicals).

α -glucosidase was from Megazyme. PD-10 columns were supplied by Pharmacia Biotech AB. [U- 14 C]sucrose and ADP-[U- 14 C]glucose were from Amersham International plc. The spectrophotometer used was a Pye Unicam SP 8-100 UV supplied by Unicam Spectronic. The scintillation counter used was a Beckman LS 6500. Optiphase 'Hisafe' 3 scintillation fluid was from Fisher Scientific UK.

2.1.3 Molecular biology materials

Kanamycin A (*Streptomyces kanamyceticus*), ampicillin (D- α -aminobenzylpenicillin), streptomycin and sulphadiazine were supplied by Sigma Chemical Co. Ltd., claforan (cefotaxime sodium) was from Rousell Laboratories Ltd. Endonuclease restriction enzymes, ligases and the Wizard Plus SV Kit were supplied by Promega U.K. Ltd., taq polymerase and extender were supplied by Advanced Biotechnologies Ltd. Phosphatase was from Amersham International plc and oligonucleotide primers were from Life Technologies, Inc. Plasmid-safe DNAase was supplied by Cambio. Bacterial plasmids pDPE102N and pJIT119 were kindly supplied by Dr Takeshi Takaha, Glico Co., Japan and Advanced Technologies (Cambridge) Ltd. respectively.

2.1.4 SDS-PAGE reagents and materials

Acrylamide / bisacrylamide was from Severn Biotech Ltd. All other reagents were from Sigma Chemical Co. Ltd. Protran nitrocellulose membrane was from Schleider & Schell. The ECL Plus™ kit and Secondary antibody was anti-rabbit Ig-G-horseradish peroxidase linked whole antibody (donkey) were from Amersham International plc. Cronex® medical x-ray film was from Sterling Diagnostic Imaging Inc., and the developer used was a Konica SRX-101A. from Amersham International plc.

2.1.5 Bacteriological and plant media

Luria-Bertani medium (litre ⁻¹)	10 g bacto-tryptone (Difco laboratories) 5 g bacto-yeast (Difco laboratories) 10 g NaCl, pH 7.0
GYT (litre ⁻¹)	1.25g bacto-yeast extract 2.5 g bacto-tryptone 100 g glycerol
MS medium (litre ⁻¹) (Murashige and Skoog, 1962)	4.4 g MS salts (Sigma) 20 g sucrose 6 g bacto-agar, pH 5.6
2 x YT (litre ⁻¹)	16g bacto-tryptone 10g bacto-yeast extract 5 g NaCl, pH 7.4

2.2 Biochemical procedures

2.2.1 Sampling of tissue

Tubers. Tubers between 20-60 g fresh weight were sliced longitudinally into 2 mm thick slices, these were immediately frozen in liquid nitrogen and stored for up to four months at -80 °C prior to analysis. Tubers with blemishes were discarded.

Leaves. Five fully expanded leaflets were removed from the five uppermost axial branches of a single plant and these five leaflets were pooled to comprise each individual sample. Sampling between different plants was performed in a reproducible manner by removing leaflets from equivalent positions from the leaves. Samples were immediately frozen in liquid nitrogen and leaf material was stored for up to two months at -80 °C prior to analysis.

2.2.2 Spectrophotometric analysis

For each assay, except for α -amylase and starch synthase, enzyme activity was linked to the reduction of NAD^+ . The resulting change in absorbance of light at 340 nm was used to calculate enzyme activity by employing the extinction coefficient of NADH which is calculated by the following equation where ϵ = extinction coefficient, l = path length and c = concentration of solution (Lowry and Passonneau, 1972).

$$\epsilon \frac{l}{c}$$

For NADH, $\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$

α -amylase was assayed by following the ability of an enzyme extract to change the absorbance of light of a coloured reagent. Starch synthase was assayed by measuring the incorporation of a radiolabelled substrate into starch. The absorption of solutions was made at 25 °C, in a temperature controlled, double beam spectrophotometer. Plastic cuvettes with a volume of 1 ml and path length of 1 cm were used. For each assay the background rate was measured in the absence of one of the substrates of the reaction and was subtracted from the actual rate in the presence of all of the substrates. Control reactions contained enzyme extract which had been heated to 100 °C for 5 min before being assayed. All assays were performed at 25 °C.

2.2.3 Enzyme activity assays

2.2.3.1 Preparation of enzyme extract

0.5-1.0 g frozen tissue was ground in liquid nitrogen in a mortar to a fine powder. 5 ml of extraction buffer containing 100 mM Hepes (pH 7.5), 10 mM EDTA, 5 mM dithiothreitol (DTT), 0.5 % (w/v) BSA and 0.1 % (w/v) polyvinyl pyrrolidone was added. Tissue to be assayed for D-enzyme activity was extracted in 20 mM Tris-HCl (pH 7.5) and 5 mM 2-mercaptoethanol. The samples were centrifuged for 15 min at 13 200 r.p.m.. The supernatant was decanted and 2.5 ml was applied to a PD-10 Sephadex G-25 column (which had been equilibrated with 25 ml extraction buffer); the eluent was discarded. A further 3.5 ml extraction buffer was applied to elute the enzyme extract. For samples to be assayed for D-enzyme activity the PD-10 column was equilibrated and the sample was eluted with Tris-HCl (pH 7.0). The extractions were performed at 4 °C.

2.2.3.2 D-enzyme [EC 2.4.1.25]

The assay for D-enzyme was based on Takaha *et al.* (1993). The ability of an enzyme extract to disproportionate maltotriose and the concomitant production of glucose was linked to NAD⁺ reduction. 60 µl enzyme extract was incubated in 8 % maltotriose, 100 mM Tris-HCl (pH 7.0) and 5 mM 2-mercaptoethanol in a total volume of 120 µl for 10 min. The reaction was stopped by boiling for 5 min. The samples were centrifuged at 13 200 r.p.m. for 3 min and 50 µl of the supernatant was assayed for glucose content.

2.2.3.3 ADPglucose pyrophosphorylase [EC 2.7.7.27]

The assay for ADPglucose pyrophosphorylase was based on the ability of an enzyme extract to produce glucose-1-phosphate (G-1-P) from ADPglucose and organic pyrophosphate (PPi). G-1-P production was coupled to the rate of NAD⁺ reduction (Smith, 1990a). 50 µl enzyme extract was added to 40 mM Hepes (pH 8.0), 10 mM MgCl₂, 0.4 mM NAD⁺, 0.024 mM glucose-1,6-bisphosphate, 1.5 mM ADPglucose, 4 U phosphoglucomutase and 1.4 U NAD⁺ dependent glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*) in a final volume of 1 ml. The reaction was started by adding PPi to a final concentration of 2 mM.

2.2.3.4 Soluble starch synthase [EC 2.4.1.21]

The assay for soluble starch synthase was based on the ability of an enzyme extract to incorporate ^{14}C from ADP-[U- ^{14}C]glucose into amylopectin (Smith, 1990b). 50 μl enzyme extract was added to 150 mM Bicine (pH 8.5), 400 mM sodium citrate, 0.1 mg amylopectin (potato) and 1.4 mM ADP-[U- ^{14}C]glucose (8.95 kBq μmole^{-1}) in a final volume of 200 μl . Samples were incubated for 30 min at 30 °C; the reaction was terminated by the addition of 1 ml methanol-KCl (75 % (v/v) methanol : 1 % (w/v) KCl). Addition of methanol-KCl resulted in the precipitation of α -glucan from ADPglucose. Samples were centrifuged for 2 min at 13 200 r.p.m., the supernatant was decanted and the pellet was washed again in methanol-KCl before resuspending in 200 μl water. Samples were mixed with 1 ml scintillation fluid and the rate of disintegration of the ^{14}C radionuclide was measured using a scintillation counter.

2.2.3.5 Starch phosphorylase [EC 2.4.1.1]

The assay for starch phosphorylase was based on the ability of an enzyme extract to produce G-1-P from amylopectin and inorganic phosphate (Pi). G-1-P production was coupled to the rate of NAD^+ reduction. 50 μl enzyme extract was added to 50 mM Hepes (pH 7.0), 5 mM MgCl_2 , 0.25 mM NAD^+ , 0.024 mM glucose-1,6-bisphosphate, 0.12 % (w/v) amylopectin (potato), 2 U phosphoglucomutase and 1.4 U glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*) in a final volume of 1 ml. The reaction was started by adding Pi to a final concentration of 4.5 mM.

2.2.3.6 α -amylase [EC 3.2.1.1]

α -amylase activity was measured by following the solubilisation of a suspension of starch azure by enzyme extract as detailed by Doelhart and Duke (1983). The solubilisation of the starch azure was measured by recording the increase in absorbance of light at 595 nm. Starch azure suspension was prepared by heating 2 % (w/v) starch azure, 100 mM Hepes (pH 7.6) and 3 mM CaCl_2 in a microwave on low power, mixing every 10 sec until the suspension became viscous. The suspension was cooled and stirred continuously prior to use. 50 μl enzyme extract was incubated in 100 mM Hepes (pH 7.5), 3 mM CaCl_2 , 100 μl starch azure suspension and 1000 U β -amylase in a final volume of 200 μl for 1 h. The reaction was

stopped by the addition of 800 μ l 16 % (w/v) trichloroacetic acid and centrifuged for 2 min at 13 200 r.p.m.. The absorbance of the supernatant at 595 nm was measured. Enzyme activities were calculated from calibration curves. For each separate experiment calibration curves were constructed to express the release of nmoles of maltose equivalents per min from known amounts of α -amylase (pig). Calibration curves were also constructed in the presence of saturating amounts of β -amylase, as β -amylase in enzyme extracts could increase the detectable activity (Doelhart and Duke, 1983).

2.2.3.7 β -amylase [EC 3.2.1.2]

β -amylase activity was followed by measuring the ability of a potato extract to hydrolyse amylopectin to maltose. The maltose produced was digested with α -glucosidase and the amount of glucose released was determined. 10 μ l enzyme extract was incubated in 0.2 M Hepes (pH 7.1), 10 % (w/v) amylopectin and 10 mM EDTA for 30 min in a total volume of 200 μ l. The reaction was stopped by heating to 100 °C for 5 min and the sample was centrifuged for 5 min at 13 200 r.p.m.. The glucose content of 150 μ l of the supernatant was assayed before and after the addition of 1 U α -glucosidase.

2.2.3.8 β -glucuronidase

The assay for β -glucuronidase activity follows the ability of an enzyme extract to cleave 4-methylumbelliferyl glucuronide (MUG) into glucuronic acid and 7-hydroxyl-4-methylcoumarin (MU). The accumulation of MU is determined by measuring its fluorescence according to Jefferson *et al.*, (1987). Approximately 1 g of frozen tissue was ground to a fine powder. 1 ml of extraction buffer containing 50 mM sodium phosphate (pH 7.5), 10 mM 2-mercaptoethanol, 0.1 % (w/v) Triton X-100 and 1 mM EDTA was added. The mixture was vortexed and then centrifuged at 13 200 r.p.m. for 5 min at 4 °C. 800 μ l of the supernatant was incubated with 200 μ l 5 mM MUG in extraction buffer at 37 °C. After 0, 5, 10, 15 and 30 min 200 μ l of reaction mixture was added to 800 μ l 200 mM Na_2CO_3 to stop the reaction. The concentration of MU was determined fluorometrically using a Perkin-Elmer LS series spectrophotometer with settings of excitation at 365 nm and emission at 455 nm.

2.2.4 Sugar assays

2.2.4.1 Preparation of sugar-containing extracts

Sucrose, fructose and glucose were extracted from tuber tissue according to ap Rees *et al.* (1977). 0.5 g frozen tuber tissue was ground to a fine powder and then resuspended in 1 ml 1.41 M perchloric acid for 10 min. The sample was centrifuged at 13 200 r.p.m. for 5 min, the supernatant was decanted and the pellet was resuspended in 1 ml 1.41 M perchloric acid and centrifuged as above. This was repeated once more and then the three supernatants were pooled, neutralised with 5 M K_2CO_3 to between pH 6.0-7.5 and centrifuged at 13 200 r.p.m. for 10 min. The supernatant was decanted and assayed for sugar content. All steps were carried out at 4 °C.

2.2.4.2 Glucose

Glucose was assayed after the method of Kunst *et al.* (1988). The assay was based on the ability of hexokinase and glucose-6-phosphate dehydrogenase to produce phosphogluconic acid in the presence of glucose and this was coupled to NAD^+ reduction. 20 μ l extract was added to 100 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 4 mM NAD^+ , 1 mM ATP and 1 U hexokinase, and the absorbance was left to stabilise at 340 nm. 0.5 U NAD^+ dependent glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*) was then added and the resulting change in absorbance was measured.

2.2.4.3 Fructose

Fructose was assayed by following the conversion of fructose to glucose-6-phosphate after the addition of phosphoglucose isomerase by coupling the reaction to the reduction of NAD^+ . After assaying for glucose (section 2.2.4.2) 0.7 U phosphoglucose isomerase was added to the same cuvette and the resulting change in absorbance was measured.

2.2.4.4 Sucrose

Sucrose was assayed by measuring the production of glucose and fructose after the addition of invertase and coupling this to the reduction of NAD^+ . After assaying for glucose and fructose (section 2.2.4.3), 30 U invertase was added to the same cuvette and the resulting change in absorbance was measured.

2.2.5 Starch

Leaves. Based on the method described by Hargreaves and ap Rees (1988) leaf tissue was incubated in 50 ml boiling 80 % (v/v) ethanol (EtOH). The insoluble material was homogenised in a mortar and extracted three more times in boiling 80 % (v/v) EtOH. The insoluble material was resuspended in water and autoclaved at 121 °C (114 kN m⁻²). The glucose released after an incubation in 2 U amyloglucosidase (*Aspergillus niger*), 20 U α -amylase (pig) and 0.2 M sodium acetate (pH 5.5) in a final volume of 1 ml for 18 h at 37 °C was determined. Controls were incubated without amyloglucosidase and α -amylase.

Tubers. 0.1 g frozen tuber tissue was ground to a fine powder and resuspended in 1 ml 45 % (v/v) perchloric acid. The sample was incubated on ice for 5 min. 9 ml distilled water was added and the sample was centrifuged at 13 200 r.p.m. for 5 min. The supernatant was decanted, frozen in liquid nitrogen and stored at -80 °C for up to two months prior to use. Starch was assayed by incubating an aliquot of starch extract with amyloglucosidase and α -amylase as described above for leaf tissue.

2.2.6 Amylose

The proportion of amylose in starch was determined according to the method described by Hovenkamp-Hermelink *et al.* (1988). The method is based on the fact that amylose and amylopectin exhibit different absorption spectra maxima, 618 nm and 550 nm respectively, in the presence of iodine. Starch from tuber or leaf tissue was ground to a fine powder and resuspended in 0.5 ml 45 % (v/v) perchloric acid. The sample was incubated on ice for 4 min. 8 ml distilled water was added, the sample was centrifuged at 13 200 r.p.m. for 5 min and the starch-containing supernatant was decanted. The absorbance of light at 550 nm and 618 nm of a solution containing 500 μ l starch extract and 500 μ l of 20 mM I₂ and 52 mM KI was measured. The proportion of amylose (as a percentage of the total starch) was calculated according to the following equation (Hovenkamp-Hermelink *et al.*, 1988):

$$[(3.5 - 5.1R) / (10.4R - 19.9)] \times 100$$

$$R = A_{618} / A_{550}$$

2.2.7 Malto-oligosaccharide content

The assay for malto-oligosaccharide content was based on the method described by Critchley *et al.* (2000). Frozen leaf tissue was ground to a fine powder and extracted in 1.5 ml 0.7 M perchloric acid for 30 min on ice. Samples were then centrifuged for 10 min at 13 200 r.p.m. at 4 °C. 1 ml of the supernatant was neutralised by the addition of 2 M KOH, 0.4 M MES and 0.4 M KCl. The precipitated potassium perchlorate was removed by centrifugation at 13 200 r.p.m. for 10 min at 4 °C. Samples were dehydrated by freeze drying. Samples were analysed by High Performance Anion Exchange Chromatography with pulse amperometric detection by Advanced Technologies (Cambridge) Ltd.

2.2.8 Iodine staining of intact leaves

Leaves were incubated in boiling water for 2 min and then decolourised by incubating in 95 % (v/v) EtOH at 80 °C. Starch was visualised by staining in iodine solution (0.67 % (w/v) I₂, 3.33 % (w/v) KI) and leaves were destained by five 10 min washes in water.

2.2.9 [¹⁴C]sucrose metabolism in developing and mature tubers

2.2.9.1 Tuber material

For developing tubers, two month old plants were gently removed from their pots and laid sideways on a plastic sheet; one tuber was uncovered from each plant; care was taken to ensure the stolon remained intact. For mature tubers, each tuber was placed inside a sealed plastic box and kept in darkness.

2.2.9.2 Supply of label

Based on the method described by Dixon and ap Rees (1980) a hypodermic needle was used to make a 15 mm x 1.5 mm bore hole midway between the apical and basal ends of a tuber. Using a Hamilton syringe the well was filled with 20 µl 0.354 mM [¹⁴C]sucrose (20.9 MBq µmol⁻¹). After 3 h unincorporated label was removed by three washes with 20 µl 0.5 mM sucrose and the bore hole was filled with 20 µl 0.5 mM sucrose. Trays containing 1 ml 20 % (w/v) KOH were placed next to each tuber. After supplying the label to developing tubers each tuber was enclosed in a sealed plastic bag and the plant root system

was covered by a black plastic sheet to minimize water loss. To sample tuber material, a hypodermic needle was used to remove cores of 15 mm x 1.5 mm, 5 mm distance from the original bore hole. Samples were taken from equivalent positions on individual tubers at each time point; the bore holes were sealed with petroleum jelly. The samples were immediately frozen in liquid nitrogen and stored at -80 °C prior to analysis.

2.2.9.3 Incorporation of ^{14}C into starch

To isolate the starch-containing fraction from each sample, tissue was homogenised using a micropestle in 1.5 ml tubes, held in racks in an icebox containing liquid nitrogen. 300 μl 80 % (v/v) boiling EtOH was added and the samples were incubated at 80 °C for 5 min. The samples were then vortexed for 1 min, centrifuged at 13 200 r.p.m. for 5 min and the soluble fraction was decanted from the insoluble starch-containing pellet. A further 200 μl 80 % (v/v) EtOH was added to the pellet and the sample was left for 5 min at room temperature. The sample was then vortexed for 1 min, centrifuged at 13 200 r.p.m. for 5 min and the soluble fraction was decanted. This was repeated once more with EtOH and a further three times with water; the soluble fractions were pooled into one fraction.

To measure the incorporation of ^{14}C into starch, the insoluble pellet was resuspended in 5 ml 0.2 M sodium acetate (pH 5.5). 2.5 ml of this suspension was incubated with 50 U amyloglucosidase (*Aspergillus niger*) and 900 U α -amylase (pig) at 37 °C for 18 h. Controls contained no amyloglucosidase or α -amylase. The glucose released from these incubations was separated from the insoluble pellet by centrifugation at 6000 r.p.m. for 10 min. The quantity of ^{14}C label in each of the fractions was determined using a scintillation counter.

2.3 Physical measurements

2.3.1 Effect of irradiance level on plant growth

Tubers were kept in the cold until they produced sprouts, all sprouts were then removed except the strongest one. Tubers were planted on the same day in identical pots of 25 cm diameter in an unheated greenhouse in April. One population of plants was grown in randomised positions in the middle of the greenhouse: the irradiance levels experienced by these plants ranged between 400-2000 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ over the period of plant growth. A second population of plants were grown in randomised positions in the same greenhouse but under a metal frame 2 m x 1.5 m x 1.5 m covered by a canopy of nylon netting. A gap of 30 cm was maintained above the floor to facilitate ventilation. Irradiance levels under the canopy ranged between 50-400 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ over the period of plant growth. Plant height was recorded at regular intervals.

2.3.2 Grafting

Tubers were stored in the cold until the sprouts on the slower sprouting low D-enzyme expressing lines were around 4-5 cm in length. Tubers were stripped of all but one sprout; the remaining sprout was then cut diagonally in half, approximately 2 cm from its base. The tuber-bearing portion (stock) was rejoined to an excised piece of sprout (scion) from a different tuber using a piece of sterile silicon tubing. Sprouts with similar diameters were matched for individual grafts. Grafted tubers were placed in plastic bags with wet Whatman paper and left in a growth room at 22 °C with 10 h light periods. The Whatman paper was re-wetted if it dehydrated. After four weeks tubers were weaned onto soil and were grown in acetate tubes for two weeks to reduce water loss through the graft. During plant growth the graft was maintained above soil level and any shoots produced below the graft, or stolons or aerial tubers formed above the graft, were removed. The plants were grown in randomised positions in an unheated greenhouse April-October. Plants which formed poor quality grafts were excluded from analysis.

2.3.3 Measurement of Dry Weight

Specific gravity values were determined by weighing tubers in water and comparing this to their weight in air. Weighing in water involved suspending tubers in a porous plastic bag from a gravimetric balance into a trough of water. Care was taken to ensure the bag did not touch the bucket during weighing. Specific gravity, dry matter and starch content were calculated from the following equations derived by von Scheele *et al.* (1937). von Scheele *et al.* (1937) derived a linear regression from experimental data with the values for $y = b + mx$; where for % dry matter, $b = 24.182 \pm 0.035$, $m = 211.04 \pm 3.33$ and $x = \text{specific gravity}$; and for % starch, $b = 17.546 \pm 0.03$, $m = 199.07 \pm 2.88$ and $x = \text{specific gravity}$.

Specific gravity (s.g.) = tuber weight in air / (tuber weight in air - tuber weight in water)

% Dry matter = $24.182 + ((211.040) \times (\text{s. g.} - 1.0988))$

% Starch = $17.546 + ((199.070) \times (\text{s. g.} - 1.0988))$

2.4 Molecular procedures

2.4.1 Small scale extraction of plasmid DNA

pDPE102N was isolated by growing 1.5 ml bacterial cultures overnight at 37°C in 2 x YT media with 50 µg/ml ampicillin. Cultures were centrifuged at 13 200 r.p.m. for 5 min, the supernatant was discarded and the pellet was resuspended in 100 µl solution I (50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA containing 2 % (w/v) RNAase). Cells were lysed by adding 200 µl solution II (0.2 M NaOH, 1 % (w/v) SDS) and left on ice for 15 min. The samples were neutralised by adding 150 µl solution III (3 M potassium acetate, 60 % (v/v) acetic acid) and left on ice for 30 min. Samples were centrifuged at 13 200 r.p.m. for 5 min, the supernatant was decanted and the pellet was incubated at 68°C for 15 min to enable RNA digestion. The DNA was precipitated in ethanol and then resuspended in 50 µl Tris-HCl EDTA (TE) buffer.

2.4.2 Large scale extraction of plasmid DNA

pJIT119 DNA was isolated using a caesium chloride (CsCl) extraction protocol. 500 ml bacterial cultures were grown overnight at 37°C in 2 x YT media containing 50 µg/ml

kanamycin. The cultures were centrifuged at 7000 r.p.m. for 5 min, the supernatant was decanted and the pellet was resuspended in 20 ml solution I (section 2.4.1.). Cells were lysed by adding 40 ml solution II (section 2.4.1.) and left on ice for 15 min. The samples were neutralised by adding 30 ml solution III (section 2.4.1.) and left on ice for 30 min. The samples were then centrifuged for 10 min at 7000 r.p.m. and the supernatant was filtered through Miracloth. Half a volume of isopropanol was added and the sample was left for 15 min. It was then centrifuged at 13 200 r.p.m. for 15 min, the supernatant was discarded and the pellet was resuspended in 10 ml TE buffer. 10 g CsCl, 100 μ l ethidium bromide (EtBr) and the DNA suspension were decanted into an ultracentrifuge tube and centrifuged at 40 000 r.p.m. for 72 h. After centrifugation a hypodermic needle was used to pierce the top of the tube to create a vent, a larger needle was inserted below the pink-staining DNA band and the DNA was drawn into a syringe. The DNA was washed six times with equal volumes of butanol and then precipitated in 2 ml distilled water and 10 ml ethanol at -20°C for 20 min. The pellet was resuspended in 500 μ l TE buffer.

2.4.3 Restriction enzyme digests

Digests were made for 2-3 h at 37°C. 2-3 μ g DNA was cut in 5 μ l buffer with 10-20 U of enzyme in a total volume of 50 μ l. Enzymes were denatured either by heating to 65°C for 10 min or removed from the DNA by a phenol-chloroform extraction.

2.4.4 Electrophoresis

Agarose gel electrophoresis was used to separate DNA according to size and charge. TBE buffer (0.045 M Tris-borate, 0.001 M EDTA) was used unless stated otherwise. Loading buffer (0.125 g bromophenol blue, 0.125 g xylene cyanol, 7.5 g ficoll, 35 ml distilled water) was added to each sample at a tenth of the sample volume.

2.4.5 Ethanol precipitation

DNA was precipitated from solution by adding two volumes of 100 % (v/v) EtOH, and 1 μ l glycogen (20 ng μ l) and then incubating at -20 °C for 20 min. The sample was centrifuged at 13 200 r.p.m. for 10 min and the supernatant was discarded. 100 μ l 70 % (v/v) ethanol was added and the sample was centrifuged at 13 200 r.p.m. for 5 min, the supernatant was

discarded and the pellet was air-dried and then resuspended in an appropriate volume of TE buffer.

2.4.6 Dephosphorylation of DNA

Phosphatases remove phosphate residues from 5' termini rendering them resistant to ligation by the action of ligases. Linearised DNA was incubated with 0.5 U shrimp alkaline phosphatase and 2 μ l phosphatase buffer in a total volume of 20 μ l at 37°C for 1 h. The enzyme was denatured by heating to 65°C for 15 min.

2.4.7 Extraction of DNA from agarose gels

DNA was extracted from gels made from 0.8 % (w/v) Type VII low melting point agarose and TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). After electrophoretic separation the gel was viewed under a longwave UV transilluminator; the DNA band was excised and incubated with 280 μ l TE buffer and 120 μ l 1 M NaCl at 68 °C for 10 min. 400 μ l phenol was added, the tube was thoroughly mixed and then centrifuged at 13 200 r.p.m. for 5 min. The DNA-containing supernatant was retained and the step was repeated. Traces of phenol were removed by washing with 400 μ l chloroform. The supernatants were combined and precipitated in one-tenth volume 3 M sodium acetate and two volumes EtOH, washed in 80 % (v/v) EtOH and resuspended in TE buffer.

2.4.8 *E. coli* electro-competent cell production

E. coli cultures were grown overnight at 37°C in 5 ml of L-broth and the appropriate antibiotics. 2 x 200 ml LB were inoculated with 200 μ l of the 5 ml culture and grown overnight at 37°C. At OD₆₀₀ 0.4-0.6 the cells were cooled on ice for 30 min, then centrifuged at 3500 r.p.m. for 15 min and the supernatant discarded. The pellet was washed twice in 100 ml ice-cold sterile 10 % (v/v) glycerol, then centrifuged at 3500 r.p.m. for 15 min. The pellet was resuspended in 0.4 ml GYT media; cells were divided into 90 μ l aliquots and stored at -80°C prior to use.

2.4.9 Electroporation into *E. coli*

Plasmid DNA was electroporated into *XI-IBLue* competent cells using a Biorad Genepulser. 40 µl competent cells were added to 5 µl plasmid DNA. The mixture was pipetted into an electroporation cuvette that had been kept on ice for 15 min. The cuvette was placed in the Genepulser chamber and electroporated at 1.5 V, 25 µFD and 800 Ω. 1 ml of 2 x YT medium was immediately added to the cuvette, the cells were transferred to a 50 ml tube and were incubated at 37 °C for 1 h. 100 µl transformed cells were plated on agar plates with 50 µg ml of the appropriate antibiotic. The plates were incubated overnight at 37°C.

2.4.10 Ligations

Ligation reactions included a fragment to vector DNA ratio of 3 : 1, 1 U of ligase and 2 µl ligase buffer in a total volume of 20 µl. Reactions were performed for 4-8 h at 16°C.

2.4.11 PCR amplification of D-enzyme cDNA containing novel restriction enzyme recognition sites

Primers were designed which contained 13 nucleotide bases homologous to the 5' and 3' ends of potato D-enzyme cDNA and 16 additional nucleotide bases encoding *SpeI* and *PstI* endonuclease restriction recognition sites at the 5' ends.

Upper strand: 5' GAT CCT GCA GAC TAG TAT TCT CAT TAT CA 3'

Lower strand: GAT CCT GCA GAC TAG TCG ACC AAA TAG TA

PCR reactions contained 25 ng DNA, 5 µl 10 x PCR buffer, 5 µl 10 mM dNTPs, 0.25 µl taq polymerase, 0.25 µl taq extender and 50 pmol each primer in a total volume of 50 µl. The reaction was incubated at: 94 °C 5 min 1 cycle, 94 °C 30 sec 40 °C 30 sec 72 °C 2 min 10 cycles, 94 °C 30 sec 50 °C 30 sec 72 °C 2 min 20 cycles, 72 °C 3 min 1 cycle.

2.4.12 PCR analysis of *E. coli* DNA

The presence of D-enzyme cDNA in DNA isolated from *E. coli* which had been electroporated with a plasmid construct was checked by performing the PCR reaction described in section 2.4.11. The orientation of D-enzyme cDNA within the plasmid

constructs was checked by PCR. Reactions were identical to those described in section 2.4.11 except only the 5' or only the 3' D-enzyme cDNA primer was used with either only the 3' CaMV 35 S promoter primer or 5' CaMV terminator primer. The sequences of the 3' CaMV 35 S promoter primer (M35SP3) and 5' CaMV (M35ST5) terminator primer are shown below:

M35SP3	AAA GCA ATG GGA TTG ATG TGA TAT
M35ST5	CCT TAT CTG GGA ACT ACT CAC ACA

2.4.13 *Agrobacterium tumefaciens* LBA4404 competent cell production

A 2 ml LB culture containing 50 µg ml kanamycin and 100 µg ml streptomycin was inoculated with a single colony of *A. tumefaciens* strain LBA4404 and grown at 28 °C. After 2 days 500 µl of the culture was used to inoculate 200 ml L-broth containing 50 µg ml kanamycin and 100 µg ml streptomycin. The cultures were grown at 28 °C until the OD₆₀₀ reached 1. The culture was incubated on ice for 20 min and then centrifuged at 4000 r.p.m. for 15 min at 4 °C. The supernatant was discarded and the pellet was washed once in one volume of 1 mM Hepes (pH 7.0), once in half a volume of 1 mM Hepes (pH 7.0) and once in 10 ml 10 % (v/v) glycerol. Finally the pellet was resuspended in 500 µl 10 % (v/v) glycerol and stored in 45 µl aliquots at -80 °C.

2.4.14 Electroporation of *A. tumefaciens*

40 µl competent cells were added to 50-100 ng plasmid DNA. The mixture was pipetted into an electroporation cuvette that had been kept on ice for 15 min. The cells were electroporated as for *E. coli* but at 2.5 V, 25 µFD and 600 Ω. 1 ml of 2 x YT was immediately added to the cuvette, the cells were transferred to a 50 ml tube and were incubated at 28 °C for 1 h. The cell culture was centrifuged, the supernatant was decanted, and the pellet was spread on agar plates containing 50 µg ml kanamycin and 100 µg ml streptomycin. The plates were incubated for two days at 28 °C.

2.4.15 Isolation of plasmid DNA from *A. tumefaciens*

Six ml of 2 x YT media containing 50 µg ml kanamycin and 100 µg ml streptomycin were inoculated with a single *A. tumefaciens* colony and the cultures were grown for two days at 28 °C. DNA was extracted as described for *E. coli*. (section 2.4.1.) except that the DNA was precipitated in two volumes of isopropanol. After drying, the pellet was resuspended in 10 µl 25 mM ATP, 25 µl Plasmid-safe DNAase buffer, 210 µl sterile water and 5 U Plasmid-safe DNAase and incubated overnight at 37 °C. The DNAase was denatured by heating to 70 °C for 30 min, DNA was precipitated with EtOH and resuspended in TE buffer.

2.4.16 Crude extraction of genomic DNA from plant leaves

Genomic DNA was isolated according to Edwards *et al.* (1991). A leaf was homogenised in a 1.5 ml tube with a micropestle for 15 sec in 400 µl extraction buffer (200 mM Tris (pH 7.5), 250 mM NaCl, 25 mM EDTA and 0.5 % (w/v) SDS). Samples were centrifuged for 5 min at 13 200 r.p.m. 300 µl of the supernatant were removed and added to 300 µl isopropanol. After 10 min samples were centrifuged for 10 min at 13 200 r.p.m., the supernatant was discarded and the precipitated DNA was air dried for 30 min. DNA was resuspended in 50 µl TE; 2.5 µl of the preparation was used in a 50 µl PCR reaction.

2.5 Tissue culture protocols

2.5.1 Propagation of stock plants

Solanum tuberosum cv. Prairie 123 and wild type Prairie plants were maintained *in vitro* on MS basal medium supplemented with 3 % (w/v) sucrose in Magenta GA7 vessels. Plants were grown at 19 °C under 17 h day length and 150 µmol m⁻² sec⁻¹ irradiance. Explants were subcultured at 3-4 week intervals. Plants derived from tip explants were transferred to 60 mm pots containing Fisons F1 compost 2-3 weeks after subculture and weaned for a week in a propagator. Two weeks later plants were potted into 130 mm pots of Fisons M2 compost for a further 3 weeks; the plants were used 4-5 weeks after weaning.

2.5.2 Co-cultivation of leaf discs

The youngest three fully-expanded leaves of a 4-5 week old potato plant were excised and surface sterilised in 8 % (v/v) Domestos™ for 10 min, then thoroughly rinsed in sterile distilled water. Leaf discs were excised using a 10 mm diameter cork borer in a sterile air environment. The leaf discs were co-cultivated by swirling for 2 min in 50 ml over night cultures of *A. tumefaciens* at OD₆₀₀ 0.7-0.8 which had been electroporated with a plant transformation construct. The leaf discs were blot dried on sterile filter paper and transferred 10 discs per plate to MS callusing medium containing 0.6 % (w/v) agar, 3 % (w/v) glucose, 2.5 mg l⁻¹ 6-benzylaminopurine (BAP) and 1 mg l⁻¹ 2,4-Dichlorophenoxyacetic acid, (2,4-D). The plates were sealed with cling film. Controls comprising non co-cultivated leaf discs were plated under the same conditions.

2.5.3 Subsequent cultivation and regeneration

Leaf discs were cultured for two days as described in section 2.4.1.2, before being transferred to callusing medium supplemented with 500 µg ml Claforan and 75 µg ml sulfadiazine (sul) for *glgC-16* plants or 500 µg ml Claforan and 50 µg ml kanamycin (kan) for Prairie plants. After 1 week the leaf discs were transferred at 5 discs per plate to MS shooting medium containing 0.6 % (w/v) agar, 3 % (w/v) glucose, 2.5 mg l⁻¹ BAP, 10 mg l⁻¹ gibberellic acid, 500 µg ml Claforan and 75 µg ml sul or 50 µg ml kan. The cultures were re-plated on fresh shoot induction medium every two weeks until shoots appeared. Shoots were then grown in a sterile jar containing 0.5 cm depth liquid MS supplemented with 3 % (w/v) sucrose and 500 µg ml Claforan. Growth room conditions were 20 °C, 16 hour day length and 150 µmol m⁻² sec⁻¹; plates were maintained in randomised positions within the growth room.

2.5.4 Microtuber production

Microtubers were induced from regenerated shoots by growing a nodal cutting with a stem diameter greater than 1 mm on MS media containing 2 mg l⁻¹ kinetin, 6 % (w/v) sucrose and ancymidol. Ancymidol was added at 9 ml per 500 ml of MS media. Four nodal cuttings were grown per 100 ml jar in darkness for 4-6 weeks at 19 °C.

2.6 SDS-Page electrophoresis

Regenerated transgenic plants were screened for D-enzyme protein expression using SDS-polyacrylamide electrophoresis (SDS-PAGE), followed by immunodetection of the D-enzyme protein using a polyclonal antibody raised against pure D-enzyme protein.

2.6.1 Extraction of protein for SDS polyacrylamide gel electrophoresis

Tuber material was homogenised in 300 µl of 20 mM Tris-HCl (pH 7.5) and 5 mM 2-mercaptoethanol. The sample was centrifuged for 5 min at 3000 r.p.m. and the supernatant was immediately assayed for protein content (section 2.6.2). All steps were carried out at 4 °C.

2.6.2 Protein determination

Protein determination was based on the method described by Bradford (1976). 10 µl protein extract (section 2.6.1) was mixed with 990 µl Bio-Rad assay reagent (diluted five-fold with distilled water) in a cuvette. After 5 min the absorbance at 595 nm was measured and protein content was calculated by reference to a standard curve of absorbances at 595 nm of known amounts of BSA. Concentrated samples were diluted before the addition of reagent to give a final absorbance in the range 0.1-0.6 at 595 nm.

2.6.3 Gel preparation

SDS-PAGE was carried out as described by Laemmli (1970). Proteins were separated on 10 x 8 cm gels using a mini gel apparatus (ABN). Gels were poured on a gel casting system (Hoefer) between glass and notched alumina plates which were separated by 1 mm wide spacers. The 10 x 6.5 cm, 10 % acrylamide resolving gel consisted of 1.5 M Tris-HCl (pH 8.8), 9.8 % (w/v) acrylamide, 0.2 % (w/v) bis-acrylamide (N,N'-methylenebisacrylamide) (30 : 0.8), 0.4 % (w/v) SDS, 0.5 % (w/v) ammonium persulphate (APS) and 0.05 % (v/v) N,N,N',N'-tetramethylene-ethylenediamine (TEMED). The 4 % 10 x 1.5 cm stacking gel consisted of 0.7 M Tris-HCl (pH 6.8), 3.9 % (w/v) acrylamide, 0.15 % (w/v) SDS, 0.08 % (w/v) APS and 0.05 % TEMED.

Protein samples of between 20-40 μg were mixed with loading buffer (60 mM Tris-HCl (pH 6.8), 25 % (v/v) glycerol, 2 % (w/v) SDS, 14.4 mM 2-mercaptoethanol and 0.1 % (w/v) bromophenol blue) in a 3:1 ratio. Prestained markers and protein samples were denatured by heating to 100 $^{\circ}\text{C}$ for 5 min. After cooling, samples were loaded onto the SDS polyacrylamide gel in volumes upto 15 μl . Gels were run in electrophoresis buffer (25 mM Tris (pH 8.3), 125 mM glycine, 0.1 % (w/v) SDS, 10 % (v/v) methanol) for 1 h at 140 V.

2.6.4 Semi-dry electroblotting of proteins onto nitrocellulose membrane

Proteins separated on SDS polyacrylamide gels were transferred to Protran BA85 nitrocellulose membrane by using a Trans-Blot SD semi-dry transfer cell (Bio-Rad). Whatman paper, nitrocellulose membrane and the SDS-PAGE gel were trimmed to appropriate sizes and equilibrated for 30 min in transfer buffer (50 mM Tris (pH 8.3), 192 mM glycine, 20 % (v/v) methanol). Four pieces of Whatman paper were placed on the Bio-Rad semi-dry electroblotter. The nitrocellulose membrane was placed on top on the Whatman paper followed by the gel and the remaining four pieces of Whatman paper. The blotter was run at 12 V for 45 min.

2.6.4.1 Immunodetection of protein on the membrane

Membranes were 'blocked' by incubating in TBS-bTween-20 buffer (25 mM Tris (pH 7.6), 0.15 M NaCl, 0.2 % (v/v) bTween-20) and 1 % (w/v) skimmed milk powder for 30 min whilst being shaken. After blocking the membrane was incubated with D-enzyme antisera (rabbit), kindly donated by Dr. Takeshi Takaha, Glico Co. Japan. The membrane was incubated in a 1/3500 dilution of antibody in TBS-bTween-20 and 1 % (w/v) skimmed milk powder overnight, at room temperature whilst being shaken. The membrane was rinsed three times in TBS-bTween-20 and then washed four times for 15 min in TBS-bTween 20. The membrane was then incubated in TBS-bTween 20 containing 1/2000 dilution of secondary antibody (anti-Ig from Donkey) for 1h. The membrane was rinsed three times in TBS-bTween-20 then washed six times for 15 min in TBS-bTween-20.

The immunodetected proteins were visualised using an ECL Plus detection kit. The membrane was exposed to X-ray film and developed. The ECL Plus system works by detecting immobilised specific antigens which are conjugated to horseradish peroxidase

labelled antibody. A series of enzymatic reactions then leads to the generation of an acridinium ester, which produces an intense light with maximum emission at 430 nm (Akharem-Tafti, *et al.*, 1995a, b).

Chapter 3. Biochemical and Growth Analysis of *Solanum tuberosum* L. cv. May Queen with Lowered D-enzyme Activity

3.1 Introduction

Transgenic potato (*Solanum tuberosum* L.) plants cv. May Queen with lowered D-enzyme activity have been engineered previously (Takaha *et al.*, 1998c). The plants were transformed with homologous D-enzyme cDNA in antisense or sense orientation linked to a CaMV 35 S promoter. Three antisense plants with D-enzyme activity lowered to less than 3 % of wild type were analysed by Takaha *et al.* (1998c). It was found that lack of D-enzyme resulted in slow plant growth, a small reduction in tuber yield and delayed sprouting of tubers. However, the starch content of tubers was not appreciably altered in amount, proportion of amylose, molecular weight of debranched amylopectin or branch chain length. The work undertaken in this chapter aimed to ascertain that these phenotypes were due to the lowering of D-enzyme activity rather than to pleiotropic effects on other enzymes. The characterisation of phenotypes associated with the lowering of D-enzyme activity in these plants was also continued.

Three independent transformants, two antisense lines, E211 and E209, and one sense line, D204, were investigated. By studying independent transformants any potential artefacts induced by somoclonal variation should be prevented from being treated as authentic phenotypes. The possibility that the introduction of a transgene into these plants may have affected non-target genes was investigated initially. Experiments to look for pleiotropic effects are warranted for two reasons. Firstly, the expression of some genes is cross-regulated. For example, in maize a mutation in the sucrose synthase gene *Sus1* is compensated for by the ectopic activation of the *Sh1* gene that encodes a second isozyme of sucrose synthase (Chourney and Taliercio, 1994). Secondly, if an enzymatic activity is lowered then the product(s) of that activity may also be lowered. This could alter the substrates available for other enzymes and possibly affect their activity or synthesis. The enzymes which were assayed in this study are involved in aspects of starch metabolism which have been linked to potential roles for D-enzyme.

Potato tubers exhibit substantial levels of biochemical variation. This has implications for the method of sampling tubers for analysis. A gradient of metabolites and enzyme activities exists along the stolon to apical axis of a tuber (Merlo *et al.*, 1993). Variation also occurs between individual tubers from the same plant (Engels and Marschner, 1986). To take account of this variation tubers were sampled along the longitudinal axis. Tubers 20-60 g fresh weight were analysed to enable comparisons between tubers that were at approximately the same developmental stage. Enzyme assays were performed under optimal conditions to enable potential differences in activities more likely to be detected. It has been reported that some enzymes are more susceptible to loss than others during extraction (Pollock and ap Rees, 1975). In this study each assay was validated by measuring the losses incurred during the freezing and extraction of tuber tissue prior to assay.

Three approaches were employed to study the effects on phenotype of a lack of D-enzyme in these plants. The first approach was to grow plants under conditions that were likely to induce particularly strong phenotypic differences, possibly stronger than have been recorded previously. It has been suggested that potato plants which lack D-enzyme exhibit a greater reduction in growth and yield relative to wild type when grown under low irradiance (Takaha *et al.*, 1998c). In this study data were accumulated from successive harvests and plants were grown under controlled light regimes to investigate how the effect of the transgene may change with growth conditions. Secondly, the effect of organ specific expression of D-enzyme activity on plant growth and yield was studied. The aim of this was to investigate whether expression of D-enzyme in source or sink tissue only would effect whole plant metabolism. A molecular approach to achieving organ specific expression of D-enzyme has been attempted previously (Takaha, 1996). Potato plants were transformed with sense and antisense orientation D-enzyme cDNA driven by a patatin promoter. The resulting plants did not include any low D-enzyme expressing lines. In this study a grafting technique was used. Lastly, the accumulation of sugars in tubers which lack D-enzyme was measured during storage. Potato tubers generally accumulate sugars after prolonged storage; this is not necessarily linked to sprouting but rather to senescence (Burton *et al.*, 1992). Senescent sweetening occurs when potato tubers are stored for a prolonged period above 10 °C. It is generally characterised by an initial period of a constant sugar content followed by a rise, the rate of which increases during the storage period (Burton, 1989). During sprout growth sucrose has been reported not to accumulate significantly, whereas reducing sugars

may accumulate transiently (Edelman and Singh, 1966; Davies and Ross, 1987; Davies and Viola, 1988). Starch degradation is thought to be the mechanism responsible for senescent sweetening and sprout growth although the exact route of starch breakdown is unclear (Burton, 1989; for review see, Davies 1990; Sowokinos, 1990a). As sprouting is delayed in tubers which lack D-enzyme they may be expected to exhibit differences in sugar accumulation compared to wild type. To investigate this the accumulation of glucose, fructose and sucrose was measured during storage at 12 °C.

3.2 Results

3.2.1 Validation and optimisation of the D-enzyme activity assay

The assay for D-enzyme activity relies on D-enzyme being the only significant source of glucose production during an incubation of enzyme extract with maltotriose. To validate this method the ability of other starch metabolic enzymes to produce glucose from maltotriose was assessed. Physiologically relevant amounts of β -amylase (sweet potato), α -glucosidase (yeast) and α -amylase (*Bacillus*) were incubated with maltotriose under the conditions normally used to assay D-enzyme. Only extremely low levels of glucose production were detected: $12.4 \pm 0.8 \text{ nmol min}^{-1} \text{ g}^{-1}$ Fresh Weight, $2.1 \pm 0.3 \text{ nmol min}^{-1} \text{ g}^{-1}$ Fresh Weight and $17.6 \pm 1.2 \text{ nmol min}^{-1} \text{ g}^{-1}$ Fresh Weight for β -amylase, α -glucosidase and α -amylase respectively. Contaminating activity from α -glucosidase and α -amylase was also assessed by incubating each enzyme with maltose or maltotriitol (reduced maltotriose). Maltose and maltotriitol are substrates for α -glucosidase and α -amylase respectively but neither substrate is metabolised efficiently by D-enzyme. The contaminating activity detected in each of these incubations was negligible: $3.2 \pm 0.7 \text{ nmol min}^{-1} \text{ g}^{-1}$ Fresh Weight and $14.8 \pm 1.6 \text{ nmol min}^{-1} \text{ g}^{-1}$ Fresh Weight for α -glucosidase and α -amylase respectively. In support of these findings the action of β -amylase on maltotriose has also been observed to be very slow in another report (Chapman *et al.*, 1972) and α -glucosidase is reported to be completely inhibited by 0.1 M Tris pH 6.8 which is present in the D-enzyme assay solution (Killilea and Clancy, 1978). In addition, the low activities of α -amylase, β -amylase and α -glucosidase compared to D-enzyme also reduces any error. It was concluded that the assay could be used as a reliable estimate of D-enzyme activity.

The assay for D-enzyme activity was optimised to enable measurement of maximum catalytic activity (figure 1). The optimum pH was 7.0, the saturating amount of maltotriose was 8 % and the assay exhibited a linear response to the amount of extract, at least up to 90 μl , and to time, at least up to 15 min. The other assays employed in this chapter were also optimised (see appendix).

Enzyme activities were measured in tubers that had been stored at 4 °C for 8 weeks. This treatment was chosen because stored tubers with lowered D-enzyme activity exhibit a particularly strong phenotype i.e. a delay in sprouting (Takaha *et al.*, 1998c). Using cold

stored tubers has two implications for the measurement of starch metabolic enzymes. Firstly, levels of starch synthetic enzymes need to be high enough to be measured. Sweetlove *et al.* (1996b) found that starch synthesis does occur in tubers stored for 8 weeks at 4°C. In addition, metabolites required for starch synthesis such as ATP and glucose 1-phosphate are present (Merlo *et al.*, 1993) and D-enzyme activity is high (Takaha, 1996) in cold stored tubers. Secondly, cold stored tubers contain high levels of glucose which could complicate the assay for D-enzyme. Although D-enzyme activity in tubers is high it was important to check the sensitivity of the D-enzyme assay. Extracts from freshly harvested tubers were divided into two halves. To one half glucose was added in an amount found in cold stored tubers. The D-enzyme activity detected in samples spiked with glucose was $98.6 \% \pm 0.3$ of unspiked samples (results not shown), indicating the assay was sensitive enough to be used on cold stored tubers.

3.2.2 The effect of freezing and the extraction process on enzyme activity

Enzyme activity could potentially be lost due to the effect of freezing tuber tissue prior to analysis or during the extraction and desalting processes. Losses due to freezing were investigated by comparing enzyme activity in extracts from equivalent samples of fresh or frozen tissue. Losses due to extraction were measured by analysing the recovery of pure enzyme added to frozen tissue prior to extraction. Experiments were also performed to investigate the recovery of sugars. Results from recovery experiments are shown in tables 1 and 2. None of the recoveries showed losses greater than 12.5 %. In addition, recoveries between wild type and transgenic tubers were not significantly different (when analysed by one-way ANOVA with a 95 % confidence limit).

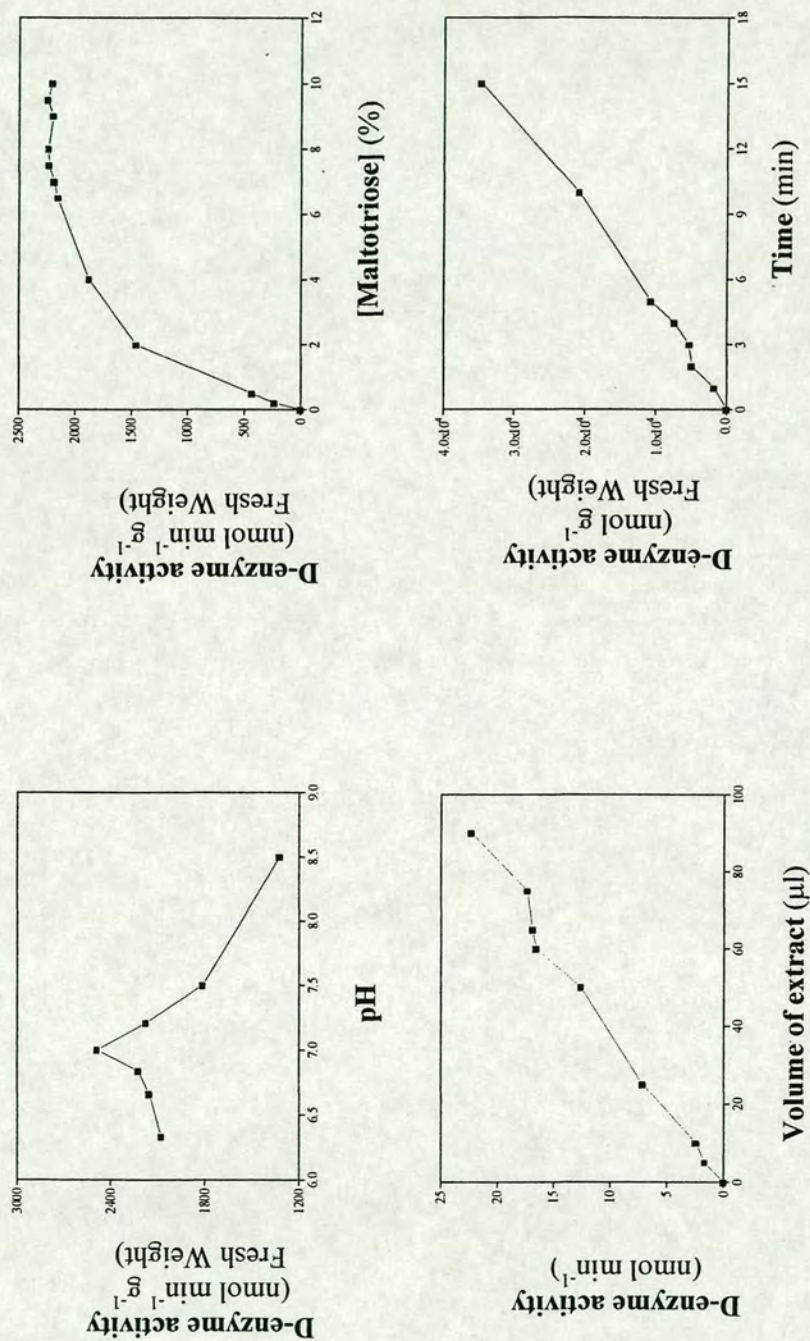


Figure 1. The optimisation of the D-enzyme activity assay in potato tuber extracts.

Potato tubers were harvested at the end of a growing season and stored for 8 weeks at 4 °C. Tubers were selected and assayed for D-enzyme activity as described in chapter 2. The assay was optimised with respect to pH, maltotriose concentration, amount of extract and the length of incubation. Parameters were varied to enable maximum catalytic activity to be measured.

3.2.3 Activities of starch metabolic enzymes in potato tubers with lowered D-enzyme activity

The possibility that the introduction of D-enzyme cDNA may have affected non-target enzymes was investigated by assaying other starch metabolic enzymes in these plants. Tubers were harvested from mature plants and stored for 8 weeks at 4 °C. Assays were performed under optimal conditions. Losses of enzyme activity due to freezing and the extraction processes were shown to be minimal (section 3.2.2). The activities of enzymes from two antisense lines, E209 and E211, and a sense line, D204, are shown in table 3. The only enzyme to show a significant difference in activity (when analysed by one-way ANOVA with a confidence limit of 99.5 %) between tubers with lowered D-enzyme activity and wild type was D-enzyme.

3.2.4 Flower colour

Wild type and the transgenic lines E211 and D204 produce plants which develop pink flowers. In contrast E209 plants produce white flowers. The flowers from the E209 plants shown in figure 2 were photographed 2 weeks after the wild type plants due to the later flowering of these plants. This phenotype has been stable through several generations (S. M. Smith, personal communication).

3.2.5 Tuber yields from four different harvests of potato plants with lowered D-enzyme activity

The tuber yield of wild type and low D-enzyme expressing plants from four separate harvests is shown in table 4. The mean tuber yield from four harvests of E209 and E211 plants compared to wild type was around 10 % and 20 % more respectively than that reported from one harvest in 1996 (Takaha *et al.*, 1998c).

Enzyme / sugar	Percentage of enzyme activity or of quantity of pure sugar, from frozen tissue relative to equivalent fresh tissue.	
	Wild type	E209
D-enzyme	93.2 \pm 2.9	94.5 \pm 0.6
ADPglc pyrophosphorylase	92.9 \pm 4.4	96.2 \pm 1.6
Starch synthase	91.2 \pm 2.7	91.9 \pm 1.3
Starch phosphorylase	90.1 \pm 3.5	94.1 \pm 1.5
α -amylase	93.8 \pm 2.6	94.3 \pm 2.2
β -amylase	97.2 \pm 1.3	95.5 \pm 1.8
sucrose	97.4 \pm 0.9	98.0 \pm 0.6
glucose	96.8 \pm 0.7	96.5 \pm 1.6
fructose	95.6 \pm 1.8	97.0 \pm 0.4

Table 1. The effect of freezing tuber tissue prior to the assay of starch metabolic enzymes and sugars.

Plants were grown April - October 1998 in an unheated greenhouse. Tubers were harvested at the end of the growing season and stored for 8 weeks at 4 °C. Tubers were selected as described in chapter 2. A longitudinal section of tuber tissue of approximately 1 g was isolated and divided into two longitudinal halves. Each half was weighed. One half was immediately frozen in liquid nitrogen and then extracted; the other half was immediately extracted. Both extracts were desalted and then assayed for enzyme activity as described in chapter 2. Each value is the mean of three separate tubers \pm s.e.m..

Enzyme / sugar	Percentage of pure enzyme activity or amount of pure sugar recovered	
	Wild type	E209
D-enzyme	93.4 \pm 7.5	92.4 \pm 3.6
ADPglc pyrophosphorylase	91.6 \pm 1.2	92 \pm 4.5
Starch phosphorylase	89.7 \pm 3.6	90.3 \pm 3.1
α -amylase	97.2 \pm 4.2	94.8 \pm 2.5
β -amylase	91.1 \pm 5.9	88.5 \pm 10.2
sucrose	95.9 \pm 1.4	95.7 \pm 2.7
glucose	94.1 \pm 2.3	97.3 \pm 1.2
fructose	93.8 \pm 1.6	97.6 \pm 0.3

Table 2. The effect of the extraction process prior to the assay of starch metabolic enzymes and sugars.

Plants were grown April - October 1998 in an unheated greenhouse. Tubers were harvested at the end of the growing season and stored for 8 weeks at 4 °C. Tubers were selected as described in chapter 2. A longitudinal section of tuber tissue of approximately 1 g was isolated and immediately frozen in liquid nitrogen. The frozen tuber tissue was divided into two halves and was weighed. A known amount of metabolite or pure enzyme (when available) of an amount or activity similar to that found *in vivo* was added to one half. Pure D-enzyme, ADP glucose phosphorylase, starch phosphorylase, α -amylase and β -amylase were from potato, *E. coli*, rabbit muscle, *Bacillus*. and sweet potato respectively. Both halves were extracted, desalted and assayed for enzyme activity as described in chapter 2. The

3.2.6 The growth of potato plants with lowered D-enzyme activity grown under low and high irradiance

Two populations of potato plants with lowered D-enzyme activity were grown under different irradiances. One population was grown in an unshaded area of a greenhouse. The irradiance levels experienced by these plants ranged between 400-2000 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ over the course of the experiment. A second population was grown under a canopy of nylon netting in the same greenhouse. The irradiance levels experienced by this population ranged between 50-400 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ over the course of the experiment. Plants grown under the canopy had an etiolated phenotype. They exhibited elongated internodes, dark coloured leaves and brittle stems. Iodine staining of leaves from these plants indicated they contained lower levels of starch than the plants grown under higher irradiance (data not shown). These features indicated the irradiance level under the canopy was low enough to affect plant growth. The growth of each set of plants is shown in figure 3. When plants were grown under high irradiance wild type plants reached a final height that was greater than any of the three transgenic lines and was significantly higher than E209 and E211 (when analysed by one-way ANOVA with a 95 % confidence limit). When grown under lower irradiance plants which lacked D-enzyme exhibited a final height that was similar to wild type. However, all plants reached the canopy roof and this is likely to have affected final plant height.

3.2.7 Tuber yields of plants with lowered D-enzyme activity grown under low and high irradiances

The yield of tubers harvested from plants which lack D-enzyme grown under different irradiances is shown in table 5. The fresh weight yield of each transgenic line relative to wild type was reduced to a greater extent when plants were grown under low irradiance. The yield of E209 plants grown under low irradiance was greatly reduced at 61 % of wild type.

Enzyme	Activity (nmol min ⁻¹ g ⁻¹ Fresh Weight)			
	Wild Type	D204	E211	E209
D-enzyme	1999 ± 45.1	41.4 ± 12.7	18.6 ± 4.0	19.4 ± 4.1
ADPglc pyrophosphorylase	128.6 ± 14.2	151.3 ± 12.4	111.7 ± 12.0	138.0 ± 17.4
Starch synthase	13.7 ± 2.2	15.1 ± 3.3	13.9 ± 0.8	15.5 ± 3.5
Starch phosphorylase	160.8 ± 13.8	163.6 ± 23.7	130.0 ± 27.1	167.0 ± 18.5
α-amylase	85.3 ± 4.2	67.2 ± 10.6	88.5 ± 11.1	69.6 ± 5.9
β-amylase	40.1 ± 7.8	45.2 ± 3.6	37.9 ± 6.9	41.2 ± 13.9

Table 3. The activities of D-enzyme and other starch metabolic enzyme in wild type and transgenic potato tubers with lowered D-enzyme activity.

Plants were grown April - October 1998 in an unheated greenhouse. Tubers were harvested at the end of the growing season and stored for 8 weeks at 4 °C. Tubers were selected and assayed for enzyme activities as described in chapter 2. Assays were performed under optimal conditions. The values are the means of three individual tubers ± the s.e.m.. Values in bold are significantly different to wild type values (analysed by one-way ANOVA with a 99.5 % confidence limit).



Figure 2. The flowers of a) wild type and b) E209 plants.

Tubers from wild type and line E209 were planted on the same day in identical pots and grown to maturity in the same greenhouse. The flowers from a) wild type and b) E209 plants were photographed one week after their emergence.

Line	Tuber yield (g)				Mean tuber yield from four harvests (percentage of wild type)
	1996 n = 3	1997 n = 4	1998 n = 4	1999 n = 5	
Wild Type	420.0 ± 122.0	989.0 ± 49.0	544.5 ± 22.6	760.8 ± 23.8	100
E211	318.6 ± 39.2	1059.3 ± 71.9	640.8 ± 61.9	668.8 ± 18.4	97.2
E209	297.4 ± 13.9	868.5 ± 57.8	493.8 ± 83.7	605.8 ± 17.4	82.2
D204	n/a	1003.8 ± 43.2	585.8 ± 33.3	743.5 ± 19.4	102.3

Table 4. The tuber yields from four consecutive harvests of wild type and low D-enzyme expressing plants.

Potato plants were grown to maturity and tubers were harvested after the foliage became necrotic. In each experiment control and transgenic lines were grown in randomised positions. Plants from 1997 and 1999 were grown April-October under natural light in an unheated greenhouse in pots of 30 cm diameter. Plants from 1996 (Takaha *et al.*, 1998c) and 1998 were grown October-April in a heated greenhouse at 20 °C, 16 h day length and at least 300 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ irradiance in pots of 20 cm diameter. Values in bold are statistically different from wild type (when analysed by t test with a 95 % confidence limit for E211 and a 99.5 % confidence limit for E209). Each value is the mean of n plants \pm s.e.m.. N/a indicates data not available.

Values for specific gravity were obtained by weighing tubers in water and comparing this to fresh weight. Specific gravity indicates tuber density and can be used to calculate percentage dry matter and starch content according to von Scheele *et al.* (1937). The range of specific gravity values from every line analysed (table 5) are typical of those found in potato (Kleinkopf *et al.*, 1987). The dry matter and starch content of plants grown under low irradiance was less than from plants grown under higher irradiance. Within each plant population there were no differences in the specific gravity, dry matter and starch contents between tubers with lowered D-enzyme activity relative to wild type E211 (when analysed by one-way ANOVA with a 95 % confidence limit).

3.2.8 Starch and amylose content of potato tubers with lowered D-enzyme activity

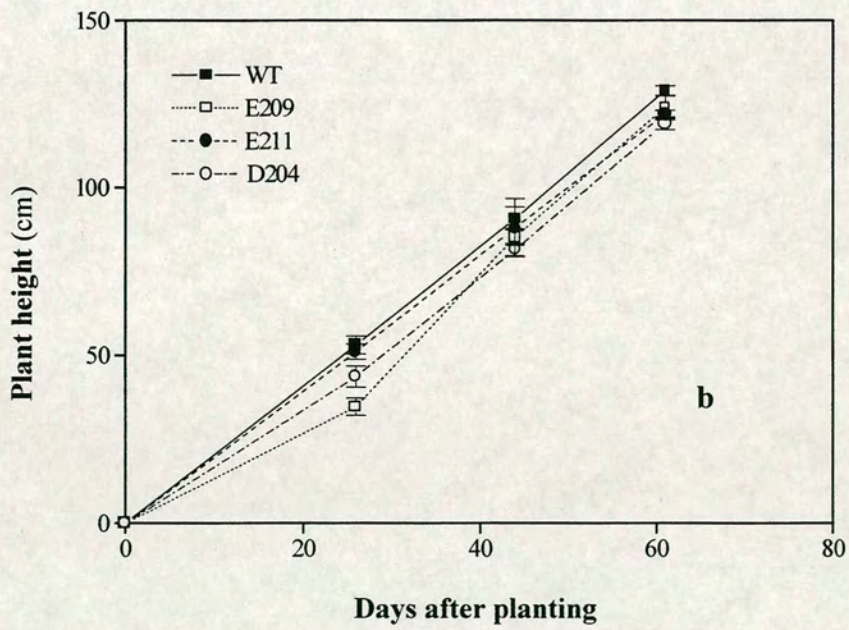
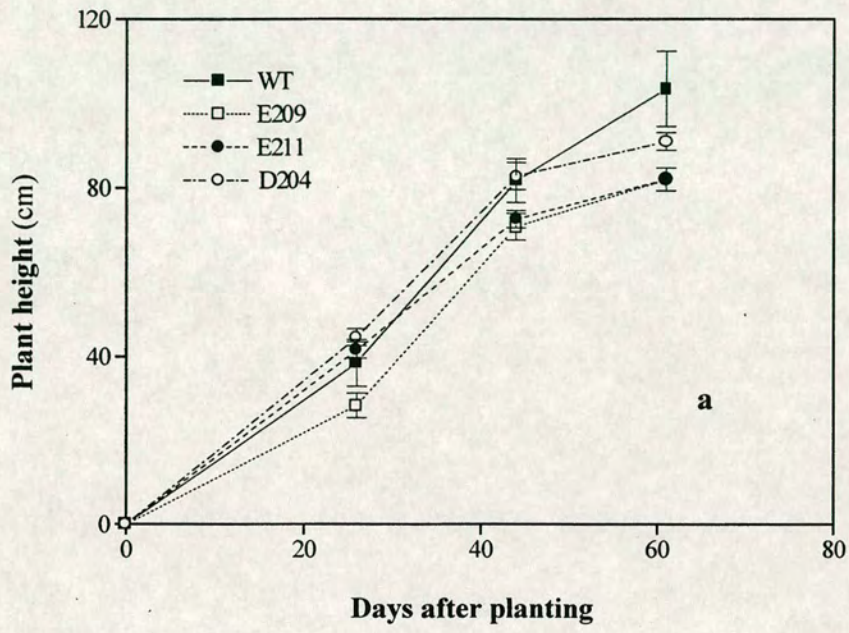
Biochemical determination of starch and amylose contents were made on freshly harvested mature tubers which had been grown in an unheated greenhouse under natural irradiance (table 6). There were no differences between the starch content or proportion of amylose between tubers with lowered D-enzyme activity and wild type (when analysed by one-way ANOVA with a 95 % confidence limit).

3.2.9 The effect on tuber yield of organ specific expression of D-enzyme activity achieved by grafting

The effect of leaf or tuber specific expression of D-enzyme activity on tuber yield was investigated by grafting sprouting tubers. Low D-enzyme expressing scions were grafted to wild type stocks and *vice versa*. Controls were same-line grafts. Tubers from line E211 were used, as the sprouts produced by tubers from D204 and E209 lines were thin and necrotic. Around 50 % of the plants survived indicating that the delicate nature of the grafts was an inherent experimental problem. The plant height and tuber yield of the grafted plants is shown in table 7. There were no significant differences in plant height, number of tubers or tuber yield between plants with leaf or tuber specific expression of D-enzyme and grafted controls (when analysed by one-way ANOVA with a 95 % confidence limit). The activity of D-enzyme in tubers and leaves originating from E211 stocks or scions was higher than has been observed previously (Takaha, 1996; section 2.2.3), the reason for this is unknown.

Figure 3. The growth of wild type and low D-enzyme expressing potato plants under a) high irradiance and b) low irradiance.

Potato plants were grown May - October 1999 in an unheated greenhouse. Two populations of plants were grown from tubers which had been kept in the cold until they produced sprouts. All sprouts were removed except the strongest one. Tubers were planted on the same day in identical pots of 30 cm diameter. One population was positioned in a designated area in the middle of a greenhouse: irradiance levels experienced by these plants ranged between 400 - 2000 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ over the duration of the experiment. A second population was grown in an identical manner but under a canopy of nylon netting, 2 m distance from the first population. The irradiance levels experienced by these plants ranged between 50-400 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ over the duration of the experiment. A gap of 30 cm was maintained at the base of the canopy to facilitate ventilation. Individual plants within each population were grown in randomised positions. Figures a) and b) show the growth of plants under high and lower irradiance respectively. The values for each time point are the mean of five individual plants. Error bars indicate s.e.m..



Line	Low irradiance					High irradiance				
	Tuber yield (g and percentage of wild type yield)	Tuber number	Specific gravity	Dry matter (%)	Starch content (%)	Tuber yield (g and percentage of wild type yield)	Tuber number	Specific gravity	Dry matter (%)	Starch content (%)
WT	331.7 ± 19.7 (100)	10.3 ± 1.7	1.076 ± 0.002	19.5	13.1	760.8 ± 23.8 (100)	18.8 ± 2.8	1.104 ± 0.002	24.4	18.5
E211	278.9 ± 33.6 (84.3)	11.8 ± 2.0	1.072 ± 0.003	18.5	12.2	668.8 ± 18.4 (87.9)	23.6 ± 3.8	1.096 ± 0.002	23.6	17.0
E209	204.1 ± 30.9 (61.5)	11.8 ± 4.4	1.079 ± 0.006	19.9	13.5	605.8 ± 7.4 (79.6)	15.8 ± 2.6	1.096 ± 0.004	23.6	17.0
D204	248.3 ± 22.9 (74.9)	8.2 ± 1.8	1.067 ± 0.004	17.5	11.2	743.5 ± 19.4 (97.7)	20.0 ± 2.1	1.096 ± 0.003	23.5	17.3

Table 5. The effect of low and high irradiance growth conditions on the tuber yield of plants with lowered D-enzyme activity.

Two populations of potato plants were grown from tubers in an unheated greenhouse May - October 1999. The tubers had been kept in the cold until they produced sprouts. All sprouts were removed except the strongest one. Tubers were planted on the same day in identical pots of 30 cm diameter. One population was positioned in a designated area in the middle of a greenhouse: irradiance levels experienced by these plants ranged between 400 - 2000 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ over the duration of the experiment. A second population was grown in an identical manner but under a canopy of nylon netting, 2 m distance from the first population. The irradiance levels experienced by these plants ranged between 50 - 400 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ over the duration of the experiment. A gap of 30 cm was maintained at the base of the canopy to facilitate ventilation. Individual plants within each population were grown in randomised positions. Values for fresh weight yield are shown in g and also as a percentage of wild type in parentheses. Specific gravity, dry matter and starch contents were measured according to the methods derived by von Scheele *et al.* (1937) which are described in chapter 2. Each value is the mean of five individual plants \pm s.e.m..

Line	Starch (mg g ⁻¹ Fresh Weight)	Amylose (%)
Wild type	93.2 ± 13.7	19.9 ± 0.8
E211	90.0 ± 12.5	20.6 ± 1.0
E209	87.9 ± 19.9	21.6 ± 1.0
D204	95.1 ± 11.0	20.7 ± 0.5

Table 6. The starch and amylose contents of wild type and low D-enzyme expressing potato plants.

Potato plants were grown in a heated greenhouse January - July 1999. Plants were grown to maturity and tubers were harvested after the foliage became necrotic. Tubers were sampled and assayed for starch and amylose content as described in chapter 2. Each value is the mean of six separate tubers ± s.e.m..

3.2.10 Accumulation of sugars in tubers with lowered D-enzyme activity stored at 12 °C

The reducing sugar and sucrose content of tubers with lowered and wild type levels of D-enzyme activity was measured at regular intervals during storage at 12 °C (figure 4). Between 7 to 17 weeks in storage the rate of accumulation of glucose and fructose occurred around four and a half times faster in wild type tubers than in E209 tubers and up to three and a half times faster than in E211 and D204 tubers. The sucrose content of wild type and transgenic tubers was constant during storage. Sprouts appeared after 7 weeks in storage on wild type tubers, after 9 weeks in storage on E211 and D204 tubers and after 10 weeks in storage on E209 tubers. The rate of sprout growth on tubers from the transgenic lines was slower than wild type; this was in agreement with a previous report (Takaha, 1996).

Plant	Final plant height (cm)	Tuber yield (g)	Number of tubers per plant	Leaf D-enzyme activity (percentage of wild type activity)	Tuber D-enzyme activity (percentage of wild type activity)
WT-WT n = 4	118.2 ± 3.5	798 .0 ± 38.3	11.5 ± 1.8	100	100
E211-E211 n = 9	99.7 ± 3.6	671.0 ± 87.1	12.7 ± 2.2	12.9	10.0
WT-E211 n = 9	109.1 ± 4.1	711.0 ± 76.3	13.3 ± 1.0	100	11.9
E211-WT n = 4	89.3 ± 4.9	814.0 ± 49.4	15.3 ± 2.3	14.5	100

Table 7. The growth and yield of grafted plants with leaf or tuber specific expression of D-enzyme activity and controls

Organ specific expression of D-enzyme activity was engineered by grafting wild type stocks onto scions from line E211 and *vice versa*. Tubers were grafted as described in chapter 2. Grafted tubers were weaned onto soil; the viability of each graft was recorded during plant growth. Grafted plants were grown April - September 1999 in an unheated greenhouse. Controls were stocks and scions which were grafted from only wild type or only E211 sprouting tubers. To calculate the relative D-enzyme activities, the D-enzyme activity in the E211 leaf or tuber tissue was expressed as a percentage of the mean of the leaf or tuber D-enzyme activities of the WT-WT control plants and the leaf or tuber wild type tissue from the respective E211-WT or WT-E211 plants. For each plant the first label indicates the scion and the second label indicates the stock from which it originated. n = the number of plants which produced successful grafts and could be used for analysis ± s.e.m..

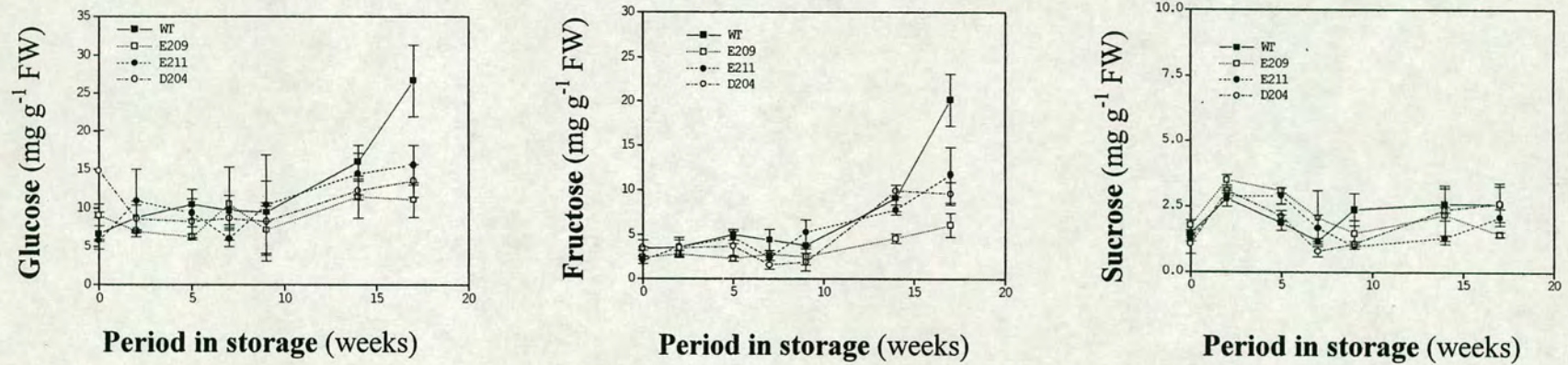


Figure 4. The accumulation of reducing sugars and sucrose in tubers from wild type and low D-enzyme expressing plants during storage at 12 °C.

Potato plants were grown in an unheated greenhouse April - October 1999. Tubers were harvested from mature potato plants and stored at 12 °C. At recorded intervals tubers were assayed for glucose, fructose and sucrose content as described in chapter 2. Each value represents the mean of three individual tubers \pm s.e.m..

3.3 Discussion

The maximum catalytic activities of a range of starch metabolic enzymes were assayed in tubers with lowered D-enzyme activity. It was shown that minimal losses of activity occurred when tissue was frozen prior to analysis and during the extraction process. There were no differences in the enzyme activities except for D-enzyme. The absence of pleiotropic effects amongst these enzymes suggests that any phenotypes detected in tubers from lines E209, E211 and D204 are likely to be due to lowering of D-enzyme activity and not to changes in these other enzymes. The aberrant flower colour of E209 indicates that either somoclonal variation or as yet unidentified pleiotropic effects have occurred in this line.

The first approach to characterise the phenotypes of these plants was to analyse the reduction in tuber yield which is associated with a lack of D-enzyme. Over four harvests the reduction in yield of the transgenic lines compared to wild type was less than had been recorded for one harvest by Takaha *et al.* (1998c). Environmental factors as well as genotype determine tuber yield (Burton, 1989). For example, high temperatures can limit yield (Burton, 1986) and water stress has been reported to inhibit starch synthesis (Geigenberger *et al.*, 1997; Geigenberger *et al.*, 1999b). Combining data from several harvests provides a better estimation of yield as it will take into account fluctuations in seasonal conditions. The yield of plants grown under different irradiances was also investigated. It has been suggested that potato plants which lack D-enzyme exhibit a greater reduction in growth and tuber yield compared to wild type when grown under low irradiance (Takaha *et al.*, 1998c). In agreement, this study found that the reduction in tuber yield of the transgenic lines was greater when grown under low irradiance rather than higher irradiance. This was despite the transgenic lines exhibiting a reduction in growth when grown under high irradiance but reaching a similar final height to wild type when grown under lower irradiance. Next, the nature of the reduction in tuber yield was investigated. The reduction in percentage dry matter and starch content of tubers from plants grown under low irradiance compared to higher irradiance is in agreement with other studies (Nash and Smith, 1939). It was found that lack of D-enzyme did not alter the percentage dry matter and starch contents of tubers from the transgenic lines relative to wild type, within either the high irradiance or low irradiance grown plant populations. Therefore, the reduction in tuber yield due to lack of D-enzyme was found to be manifested as a fall in the fresh weight yield of tubers.

There are several potential explanations which could account for the effect of lowered D-enzyme activity on tuber fresh weight yield. If D-enzyme is involved in tuber starch synthesis then lowering its activity could reduce the accumulation of starch in tubers. The fact that the starch present in the tubers of these plants appears to be normal does not support this argument. In addition, reductions in tuber yield due to lowering of starch synthetic activities within tubers have frequently been reported to alter tuber composition. For example, lowering of sucrose synthase activity in tubers through antisense technology was found to result in a reduction in tuber dry matter content whilst tuber fresh weight was unchanged (Zrenner *et al.*, 1995). Alternatively, D-enzyme in leaves could affect tuber yield. In support of this, the extent of the fall in yield due to lack of D-enzyme was shown to be affected by irradiance. Irradiance directly affects leaf metabolism but has a secondary effect on tuber metabolism. Previous studies on D-enzyme in potato have tended to interpret results in terms of tuber metabolism. There are several ways in which D-enzyme in leaves could affect tuber yield. Potato plants which lack D-enzyme generally exhibit a reduction in growth relative to wild type (Takaha *et al.*, 1998c; section 3.2.6). If this affects the photosynthetic capacity of these plants then this could affect tuber yield. However, when plants lacking D-enzyme were grown under low irradiance the tuber yield was reduced relative to wild type, despite the fact the plants reached the same final height as wild type plants. This suggests that a factor other than the effect of lack of D-enzyme on shoot growth contributed to the fall in yield. If lack of D-enzyme results in an alteration in the synthesis or mobilisation of leaf starch this could affect the supply of carbon to tubers and this could affect tuber yield. If this was the case then tuber yield would be reduced due to a fall in the supply of carbon but tuber composition would be normal providing D-enzyme was not involved in tuber starch synthesis. Alternatively, lack of D-enzyme could alter the composition of malto-oligosaccharides (MOS) in leaf tissue. In the D-enzyme mutant of *A. thaliana*, MOS metabolism is perturbed. Oligosaccharides can display activity as signalling molecules and have been implicated in the regulation of plant development (Albersheim *et al.*, 1983). Therefore it is conceivable that changes in MOS could alter metabolism in other parts of the plant including tuber metabolism. However, these theories do not provide an explanation as to why the extent of the reduction in tuber yield was greater under low irradiance, despite the fact that the plants grew to the same final height as wild type. It is possible that D-enzyme activity was altered in low irradiance grown plants due to

changes in leaf metabolism. Irradiance has been shown to alter leaf composition and the distribution of enzymatic control in tobacco (Lauerer *et al.*, 1993). Another explanation is that the expression of the transgene may have been affected by growth conditions. For example, the expression of a CaMV 35 S promoter has been shown to vary between potatoes grown under field and *in vitro* conditions (Kuipers *et al.*, 1994). As these suggestions are speculative the next approach was to investigate the role of D-enzyme in leaves and tubers separately.

It should be mentioned that the slow sprouting phenotype of low D-enzyme expressing plants introduces implicit problems in experiments designed to analyse growth and yield. It is not possible to plant wild type and transgenic tubers at stages of sprout growth which are strictly comparable. Mobilisation of tuber reserves contributes to sprout growth for a period after soil emergence (Moorby, 1978). Therefore, low D-enzyme activity is also likely to retard plant establishment, although this could also be argued as a phenotype caused by lack of D-enzyme. Generally, the rate of plant growth of the transgenic lines as indicated by plant height appears slower throughout the growth period, implying that D-enzyme is required for shoot growth (Takaha *et al.*, 1998c; section 3.2.6). The slower growth of the transgenic lines does mean that they will experience slightly different growth conditions to wild type at comparable stages of development.

It was suggested above that the reduction in yield of plants which lack D-enzyme could be due to differences in rates of leaf starch synthesis or mobilisation which could effect the supply of carbon to tubers. This was investigated by studying plants which exhibit organ specific expression of D-enzyme activity. It is not known if D-enzyme from leaves and tubers is the same isoform or if D-enzyme has a similar role in each tissue. There were no differences between the fresh weight tuber yields of plants with leaf or tuber specific expression of D-enzyme compared to control plants. The growth of plants with leaf specific expression of D-enzyme approached that of controls but this did not result in an increase in yield. These data do not necessarily indicate that the D-enzyme from leaves and tubers exerts a similar influence on plant metabolism. It is possible that the grafting method was not reliable enough to detect differences between the plants. The fact that D-enzyme activity in tubers and leaves originating from E211 tissue was higher than has been observed previously cannot be readily explained. The interpretation of results from grafted plants need to be made

with caution. It has been reported that proteins and RNA can move within phloem tissue. For example, in tobacco cosuppression of nitrate reductase has been shown to be transmitted from stock to scion tissue (Palauqui *et al.*, 1997; Xoconostle-Cazares *et al.*, 1999).

Lastly, the accumulation of reducing sugars in tubers stored at 12 °C was investigated. It is likely that rates of starch mobilisation regulate sugar accumulation (for review see Davies, 1990), although other factors such as the capacity of sprouts for growth may also be important determinants (Davies and Ross, 1987). It was shown that reducing sugars accumulated in tubers with lowered D-enzyme activity several-fold slower than in wild type tubers. The accumulation of sugars during storage at 12 °C could be due to either tuber senescence or sprouting, or a combination of both. Sprouting is reported to be a more reliable indication of starch mobilisation than reducing sugar accumulation. The accumulation of reducing sugars in sprouting tubers is relatively small compared to the amount of starch lost (Edelman and Singh, 1966; Coleman and King, 1984; Davies and Ross, 1987). The reduction in rate of sugar accumulation during storage could either be a secondary effect of the delay in sprouting of tubers which lack D-enzyme or could result from a delay in the onset of senescent sweetening.

The reduction in rate of sugar accumulation, delay in tuber sprouting and reduction in tuber yield of plants which lack D-enzyme support a role for the enzyme in the mobilisation of starch. However, the reduction in tuber yield can also be interpreted in terms of a role for D-enzyme in leaf and tuber starch synthesis. It is not known why the reduction in yield of plants lacking D-enzyme relative to wild type was greater when plants were grown under lower irradiance. The results presented in this chapter indicate the importance of studying D-enzyme in leaf as well as tuber starch metabolism. An investigation of the specific role(s) of D-enzyme in leaf and tuber starch metabolism is reported in the following chapter.

Chapter 4 Analysis of the Function of D-enzyme in Leaf and Tuber Starch Metabolism of *Solanum tuberosum* L. cv. May Queen

4.1 Introduction

Two different models have been put forward to explain the function of D-enzyme in starch metabolism. Colleoni *et al.* (1999b) proposed that D-enzyme has a direct role in starch synthesis by transferring malto-oligosaccharides (MOS) to amylopectin and so contributing to the fine structure of the amylopectin molecule. In agreement, D-enzyme mRNA has been found to accumulate during periods of rapid starch synthesis in potato (Takaha *et al.*, 1993). Other groups have suggested the primary role of D-enzyme is in starch degradation (Lin and Priess, 1988; Kakefuda and Duke, 1989; Takaha *et al.*, 1998c; Critchley *et al.*, 2000). It has been proposed that D-enzyme disproportionates smaller MOS into larger MOS which can be metabolised by other starch degrading enzymes such as starch phosphorylase and β -amylase. In potato plants which lack D-enzyme the observed delay in tuber sprouting and reduction in plant growth is consistent with a function for the enzyme in starch mobilisation (Takaha *et al.*, 1998c; chapter 3). In the previous chapter it was found that irradiance levels effect tuber yield indicating that D-enzyme in leaves may play a significant role in whole plant metabolism. In addition, an *A. thaliana* D-enzyme knock-out mutant was reported to exhibit a reduced capacity to degrade leaf starch during darkness (Critchley, *et al.*, 2000). Together, these results highlight the importance of studying D-enzyme in leaves as well as in tubers. The aim of the work in this chapter was to relate the phenotypic characteristics of potato plants lacking D-enzyme to the exact role of D-enzyme *in planta*.

Firstly, the metabolism of MOS in leaves was investigated. The approach to this was based on experiments performed on D-enzyme mutants from *A. thaliana*, *dpe1-1*, and *C. reinhardtii*, *stall1-1*, (Critchley *et al.*, 2000; Colleoni *et al.*, 1999a). During starch breakdown in the dark a substantial accumulation of MOS, primarily comprising maltotriose, was observed in *dpe1-1* (Critchley, *et al.*, 2000). This supports a direct role for D-enzyme in the

metabolism of MOS during starch degradation. In addition, *stall-1* was reported to exhibit high levels of MOS when grown under conditions which promoted starch accumulation, but the authors argued this was due to a perturbation of starch synthesis (Colleoni *et al.*, 1999a; Ball *et al.*, 1990). To our knowledge there are no other reports of the analysis of MOS from potato. The protocol employed to assay MOS in *A. thaliana* (Critchley *et al.*, 2000) was adapted for use in potato. The assay was validated by analysing the recovery of known amounts of pure MOS.

Next, transitory starch metabolism in leaves was investigated. In *dpe1-1* it was shown that rates of leaf starch degradation were reduced during the diurnal cycle, whilst rates of starch synthesis were not directly effected by lack of D-enzyme (Critchley *et al.*, 2000). *Stall-1* was reported to exhibit a reduction in starch content and altered starch structure (Colleoni *et al.*, 1999b). Iodine staining and chemical analysis were used to analyse the starch content of potato leaf tissue which lacks D-enzyme during light and darkness. The starch content of leaflets from the same plant is highly variable (Takaha *et al.*, 1998c). To compensate for this each sample comprised of five leaflets pooled from a single plant. Leaflets were sampled reproducibly by selecting one fully expanded leaflet from the five uppermost axial branches of each plant.

Lastly, starch metabolism in tubers was investigated. Rates of starch synthesis and starch degradation can be monitored by feeding [^{14}C]sucrose to intact tubers and monitoring the metabolism of ^{14}C over a pulse-chase time period. ^{14}C is incorporated into starch during the pulse. The label is then washed out and replaced with unlabelled sucrose. During the chase the rate at which ^{14}C is lost from starch can be determined. The unchanged starch content of tubers which lack D-enzyme (Takaha *et al.*, 1998c) does not necessarily mean that rates of starch synthesis and degradation are also unchanged. Several reports describe altered rates of starch turnover concomitant with unchanged starch contents (Geigenberger *et al.*, 1994; Sweetlove, *et al.*, 1996b; Trethewey *et al.*, 1999). In this study, potential changes in rates of starch synthesis and degradation were investigated. Starch synthesis was analysed in developing tubers which characteristically exhibit a net accumulation of starch. Starch degradation was analysed in sprouting tubers which characteristically exhibit a net degradation of starch. Labelling experiments using intact tubers have been shown to be an effective way of analysing starch metabolism (e.g. Merlo *et al.*, 1993; Sweetlove *et al.*,

1996a). Feeding radiolabel to tuber discs can also be used (e.g. Geigenberger and Stitt, 1993; Geiger *et al.*, 1998) but this is not suited to use over long time courses and is also reported to alter the rate and direction of metabolism (ap Rees and Beevers, 1960).

It is important to be aware of inherent problems associated with pulse-chase labelling experiments. Firstly, removal of tissue will inevitably result in wound effects. The use of this method was justified by finding that the fate of metabolised ^{14}C label was consistent over separate pilot experiments and the distribution of ^{14}C between soluble, starch and insoluble fractions was in agreement with other reports (e.g. Dixon and ap Rees, 1980; Sweetlove, 1996). In addition, wound effects in potato tubers are reported to be restricted to the two or three layers of cells next to the cut surface (Laties, 1962). By using 1 mm bore holes to supply the label and sample tissue the effects of wounding should be contained within the immediate area around each sample site. Secondly, the experimental system can only provide minimum estimates of rates of starch synthesis and degradation. The occurrence of starch turnover i.e. the breakdown of starch during net synthesis enables only net rates of starch synthesis to be estimated. Rates of starch degradation may also be underestimated if ^{14}C label released from starch is reincorporated back into starch. Thirdly, if differences in rates of starch synthesis and degradation are small they may be masked by variation between tubers. Lastly, as the exact pathway of ^{14}C sucrose into starch is unknown and internal pools exhibit large differences in intermediate concentrations and turnover, the label will be metabolised differently by individual tubers.

The aim of the work described in this chapter was to study the effect of lack of D-enzyme on MOS and starch metabolism in leaves after exposure to darkness and light. Net rates of starch synthesis and degradation were analysed in developing and sprouting tubers which lacked D-enzyme. The results were discussed in relation to the different models which have been proposed to explain the role of D-enzyme *in planta*.

4.2 Results

4.2.1 The effect of lowered D-enzyme activity on the malto-oligosaccharide content of leaves during darkness and light

Nine-week old wild type, E211 and E209 plants were maintained in a growth room in continuous darkness. Leaf tissue was sampled at regular intervals over a 30 h period. The plants were maintained in darkness for a further two days before being transferred to continuous light. Leaf tissue was sampled again at regular intervals over a 30 h period. Malto-oligosaccharide (MOS) extracts were prepared from each sample. The samples were analysed by Dr Steve Coates at Advanced Technologies (Cambridge) Ltd. using High Performance Anion Exchange Chromatography (HPAEC); results are awaited. The efficiency of recovering MOS by this method was checked by analysing the recoveries of known amounts of pure MOS. The recoveries of pure maltose, maltotriose and maltoheptaose were $98.4 \pm 0.4 \%$, $97.3 \pm 0.8 \%$ and $73.4 \pm 1.4 \%$ respectively.

4.2.2 The effect of lowered D-enzyme activity on starch breakdown during continuous darkness

Leaf tissue was sampled at the same time points during darkness from the same potato plants that were used for MOS analysis during darkness in section 4.2.1. Samples were assayed for starch content (figure 1). At the onset of the dark period the mean starch content of leaflets from line E211 was 14 % greater than wild type ($46.0 \pm 9.1 \text{ mg g}^{-1} \text{ FW}$ compared to $39.6 \pm 5.7 \text{ mg g}^{-1} \text{ FW}$). Differences in the rates of leaf starch breakdown in wild type and E211 plants were small between each of the time points. Between 0 and 5 h in darkness the average rate of starch breakdown in leaflets from line E211 was approximately 10 % slower than that of wild type. After 5 h in darkness leaflets from line E211 degraded starch at a similar or faster rate than wild type. At each time point leaf tissue from E211 contained more starch than that of wild type. Plants from line E209 grew poorly and were excluded from analysis. Pilot experiments had previously indicated the pattern of starch breakdown in E209 was similar to E211.

4.2.3 The effect of lowered D-enzyme activity on leaf starch synthesis during continuous light

Leaf tissue was sampled at the same time points during light from the same potato plants that were used for MOS analysis during light in section 4.2.1. Samples were assayed for starch content (figure 2). Differences in the rates of starch synthesis of wild type and E211 plants were small between each of the time points. Up to 8 h in light there were no appreciable differences in the rates of starch synthesis between E211 and wild type plants. Up to 17 h in light E211 synthesised starch at a slightly slower rate than wild type, although after this the rate of starch synthesis in E211 was greater than that of wild type. The variation between samples was lower than the variation exhibited by the same plants during starch breakdown (section 4.2.2).

4.2.4 The effect of lowered D-enzyme activity on the starch content of dark and light grown plants as detected by iodine staining

The effect of lowered D-enzyme activity on the starch content of leaves during light and darkness was investigated by iodine staining. Leaf tissue was sampled from plants that had been grown for 12 h in light. The same plants were maintained in darkness for 12 h and leaf tissue was sampled again. Leaflets were decolourised by boiling in 80 % ethanol and then stained with iodine to detect starch (figure 3). For each line, the leaflets from light grown plants stained darkly indicating the presence of high quantities of starch. Leaflets from wild type and D204 plants exposed to 12 h of darkness showed a substantial reduction in starch content indicated by very low staining intensity; many leaflets did not stain at all. After 12 h of darkness around two thirds of the leaves from lines E211 and E209 stained more intensely than wild type and D204.

4.2.5 The effect of lowered D-enzyme activity on the proportion of amylose in leaf starch

The effect of lowered D-enzyme activity on leaf amylose content was investigated in three separate experiments (table 1). In experiment 1 leaflets were sampled from 14 week-old plants which were flowering. In these plants the proportion of amylose in leaf starch from plants with lowered D-enzyme activity was significantly higher than wild type (when analysed by one-way ANOVA with a confidence limit of 99.5 %). In experiments 2 and 3

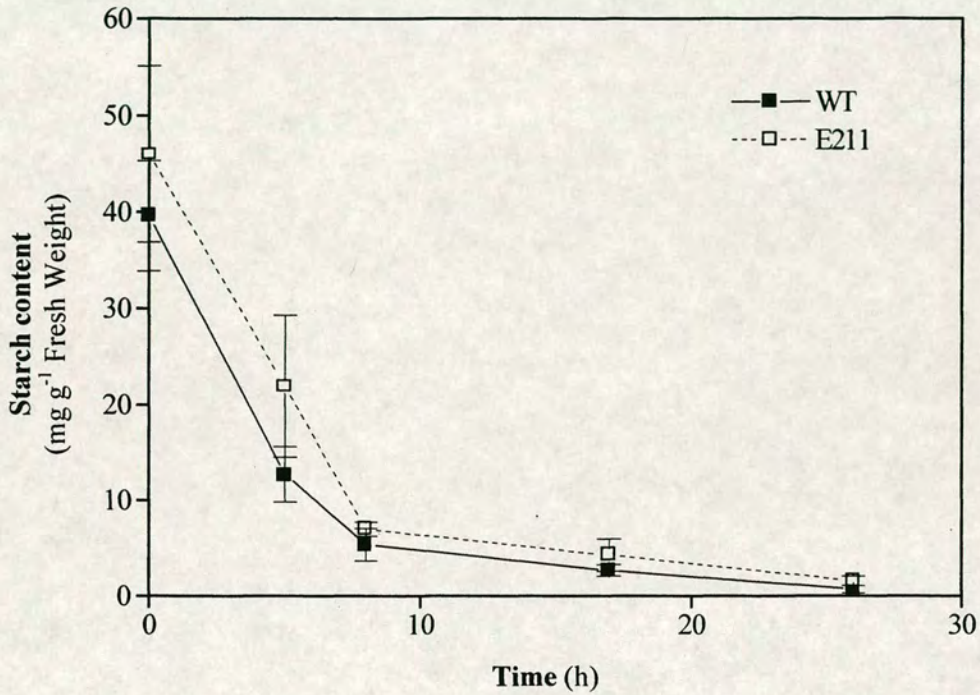


Figure 1. The leaf starch content of plants with lowered D-enzyme activity and wild type during exposure to prolonged darkness.

Seven week old potato plants were grown in random positions in a growth room under 18 h day length, 120 - 180 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance and 18 °C. Plants were maintained in the growth room for four days before the start of the experiment to acclimatise. The plants were then grown in continual darkness at 18 °C. At regular intervals leaf tissue was sampled and assayed for starch content as described in chapter 2. Each value is the mean of three plants (five leaflets were pooled from each plant for each sample). Error bars indicate s.e.m..

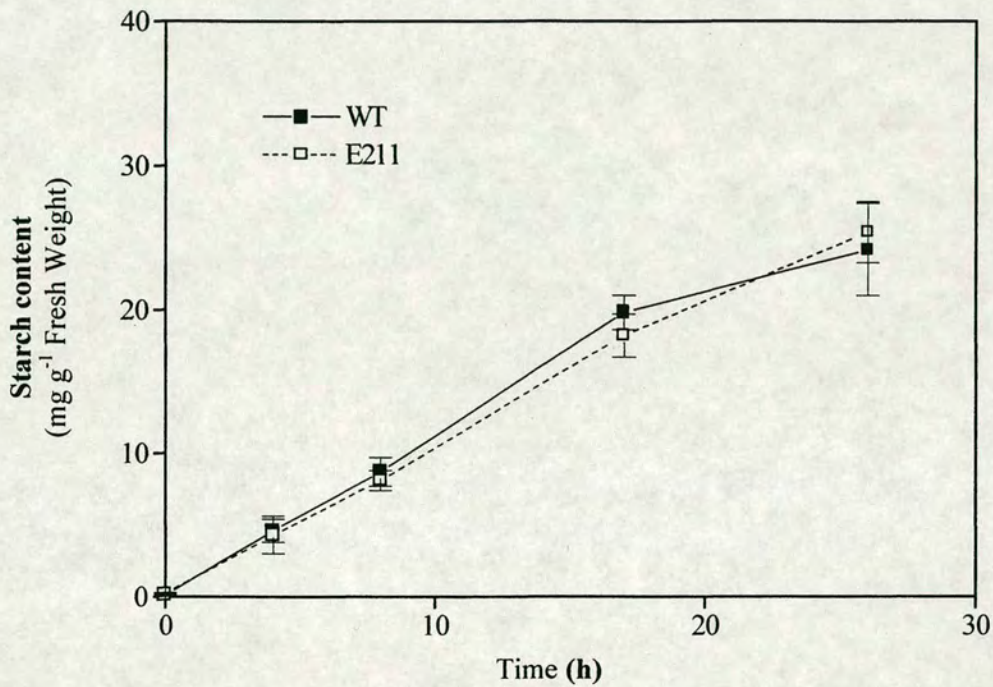


Figure 2. The leaf starch content of plants with lowered D-enzyme activity and wild type during exposure to prolonged light.

Seven week old potato plants were kept in darkness for a further two days after the end of the experiment described in section 4.2.2. The plants were then transferred to continuous light at $120 - 180 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance and 18°C . At regular intervals leaf tissue was sampled and assayed for starch content as described in chapter 2. Each value is the mean of three plants (five leaflets were pooled from each plant for each sample). Error bars indicate s.e.m..

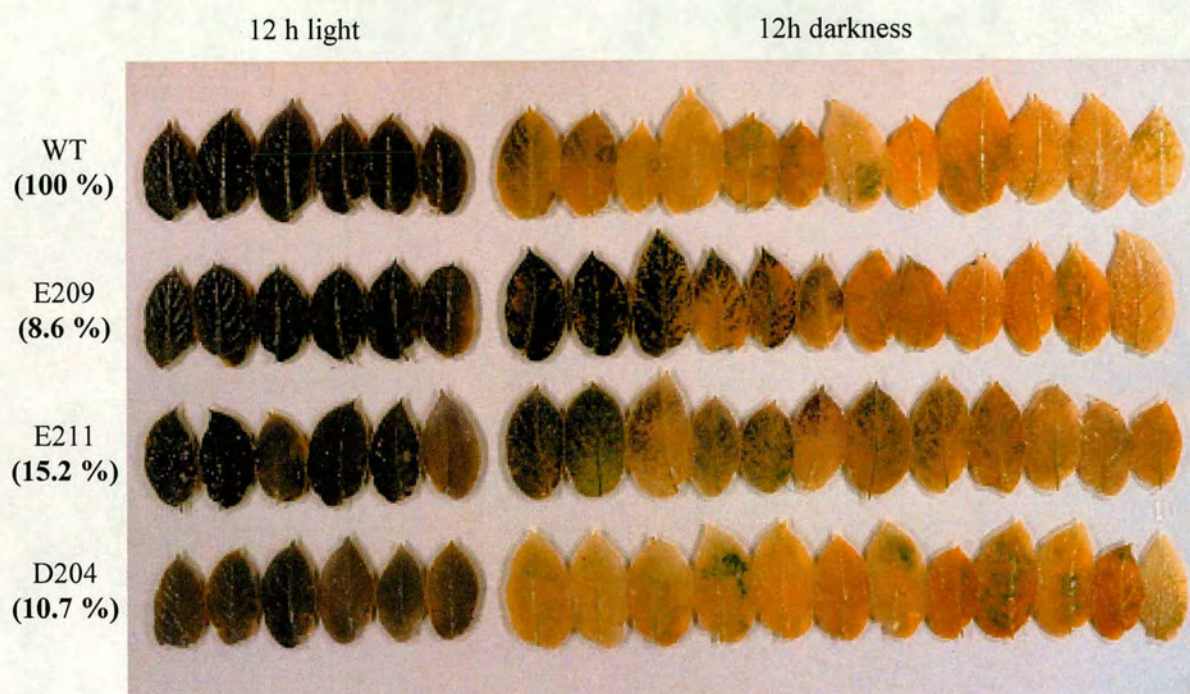


Figure 3. The starch content of leaflets from plants with wild type and lowered levels of D-enzyme activity grown in light and darkness as detected by staining with iodine.

Six week old plants were grown in random positions in a growth room under 12 h day length, $120 - 180 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance and 19°C . Plants were maintained in the growth room for four days before the start of the experiment to acclimatise. Leaf tissue was sampled and stained with iodine to detect starch as described in chapter 2. Three plants were grown from each line. Two leaflets from each plant were sampled after 12 h in light, four leaflets from each plant were sampled after 12 h in darkness. Leaf D-enzyme activity was measured for each plant after 12 h in darkness. D-enzyme activity as a percentage of wild type is shown in parentheses. The values are the mean of three plants (five leaflets were pooled from each plant for each sample).

leaf tissue was sampled from 4 and 8 week-old plants respectively. In these plants the proportion of amylose in leaf starch from plants with lowered D-enzyme activity was not significantly different from wild type (when analysed by one-way ANOVA with a confidence limit of 95 %).

4.2.6 Validation of the [^{14}C]sucrose labelling experiment

The [^{14}C]sucrose labelling experiment was validated with respect to the supply of radiolabel to tubers and the metabolism of label after the pulse chase. [^{14}C]sucrose was supplied to tubers at high specific activity and low concentration to minimise perturbations to metabolism. The delivery of label and length of the pulse have been optimised previously (Dixon and ap Rees, 1980; Sweetlove *et al.*, 1996b). To account for the basal-apical gradient of metabolites and enzymes within tubers (Merlo *et al.*, 1993) samples were taken from equivalent positions on each tuber for every time point. The incorporation of label into starch was expressed as a percentage of total label recovered to account for differences in uptake between samples. The unchanged sucrose content of the transgenic lines (chapter 3) indicated that feeding sucrose would not result in dilution effects. To validate analysis of metabolised label the fractionation of ethanol / water soluble fractions from insoluble fractions was optimised. The least number of washes required to prevent the release of additional radiolabel into solution was three with 80 % ethanol, then three with water (data not shown). Next the determination of label in starch was investigated. It has been shown previously that boiling tuber tissue in ethanol is an efficient method to enable complete recovery of starch (Dixon and ap Rees, 1980). Here it was shown that [^{14}C]starch was completely digested after an 18 h digestion with α -amylase and amyloglucosidase as no additional ^{14}C was released from incubations longer than 18 h (data not shown). It has been shown previously that 96 ± 1 % of the ^{14}C released after a digestion with α -amylase and amyloglucosidase is glucose (Sweetlove, 1996).

Line	Amylose (%)		
	Experiment 1	Experiment 2	Experiment 3
WT	6.26 \pm 0.4	16.4 \pm 0.3	12.4 \pm 0.4
E211	12.6 \pm 1.3	15.9 \pm 0.9	10.8 \pm 1.0
E209	12.2 \pm 0.2	16.4 \pm 0.6	10.8 \pm 0.9
D204	9.1 \pm 0.4	16.9 \pm 0.2	11.8 \pm 1.0

Table 1. The effect of lowered D-enzyme activity on the amylose content of leaf starch.

Leaf tissue was sampled at the end of the day and the proportion of amylose was determined as described in chapter 2. In experiment 1 leaf tissue was sampled from 14 week-old plants which were flowering and which were grown February - August 2000 in a heated greenhouse. In experiment 2 leaf tissue was sampled from 4-week old plants. In experiment 3 leaf tissue was sampled from the same plants as in experiment 2 but when the plants were 8 weeks old. These plants were grown May - October 2000 in an unheated greenhouse. Each value is the mean of three plants (five leaflets were pooled from each plant for each sample) \pm s.e.m..

4.2.7 The incorporation of [U¹⁴C]sucrose into starch in tubers with lowered D-enzyme activity

[U¹⁴C]sucrose was delivered via a cylindrical bore hole into wild type and E209 developing tubers which were still attached to a mother plant. The tubers were left for a 3 h pulse period before unincorporated label was washed out and replaced with unlabelled sucrose. Samples were removed parallel to the original bore hole at a constant distance of 5 mm. The incorporation of radiolabel into starch was determined for each sample (figure 4). There were no appreciable differences in the amount or pattern of incorporation of [U¹⁴C]sucrose into starch between wild type and E209 tubers. Between 0 h and 120 h into the chase the incorporation of label into starch increased in both lines. This was similar to the pattern of incorporation of [U¹⁴C]sucrose into starch in developing tubers that was described by Sweetlove *et al.* (1996b). The variation amongst both sets of tubers was high.

The experiment was repeated on mature tubers which had been stored for 14 weeks at 4 °C. At this stage wild type tubers exhibited sprouts of an average 30 mm length whereas E209 tubers had not started to sprout. The incorporation of radiolabel into starch was determined for each sample (figure 5). There were no appreciable differences in the amount or pattern of incorporation of [U¹⁴C]sucrose into starch between wild type and E209 tubers. The amount of label incorporated into starch was generally less than for developing tubers. For both lines between 0 h and 120 h into the chase there was an increase in label into starch. After 120 h the level of label in starch fell. This is similar to the pattern of incorporation of [U¹⁴C]sucrose into starch in mature tubers that was described by Sweetlove *et al.* (1996b). The variation amongst both sets of tubers was high.

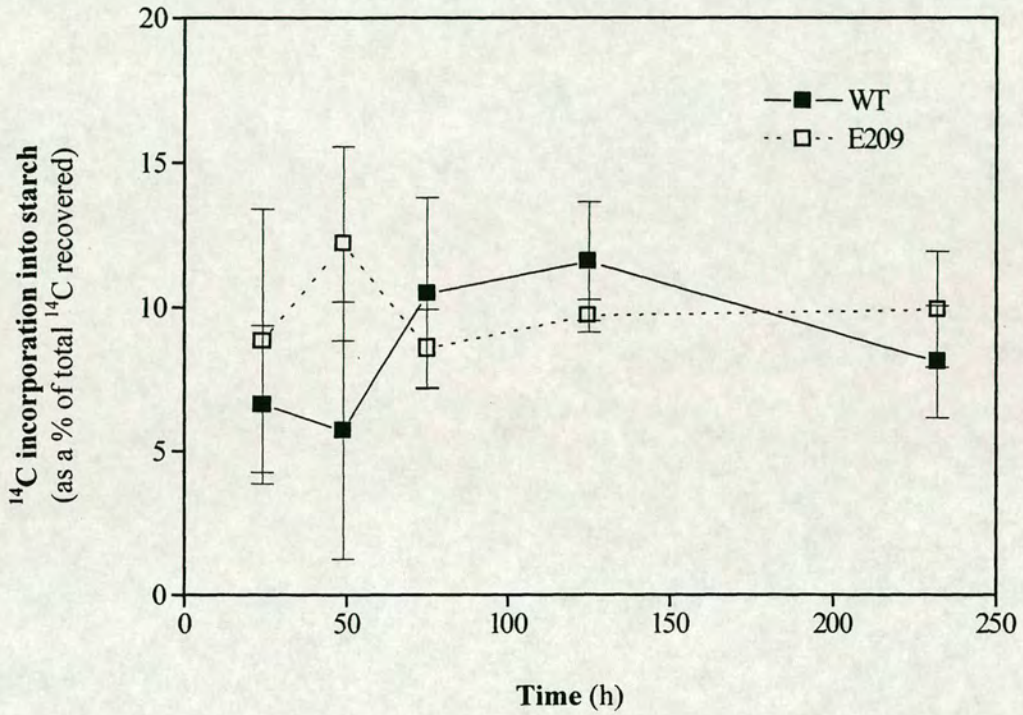


Figure 4. The incorporation of $[\text{U}^{14}\text{C}]$ sucrose into starch after a 3 h pulse in wild type and E209 developing tubers which are still attached to a mother plant.

Potato tubers were planted April 2000 in a heated greenhouse at 20 °C, 16 h day length and at least $300 \mu\text{mol m}^{-2} \text{sec}^{-1}$ irradiance. $[\text{U}^{14}\text{C}]$ sucrose was delivered to developing tubers from 10-week old plants through a bore hole midway between the apical and basal ends of each tuber. The radiolabel was left in the tuber for 3 h, then thoroughly washed out and replaced with unlabelled sucrose for the chase. Tuber tissue was sampled as described in chapter 2. The incorporation of label into starch was expressed as a percentage of the total label recovered. Each value is the mean of three individual tubers. Error bars indicate s.e.m..

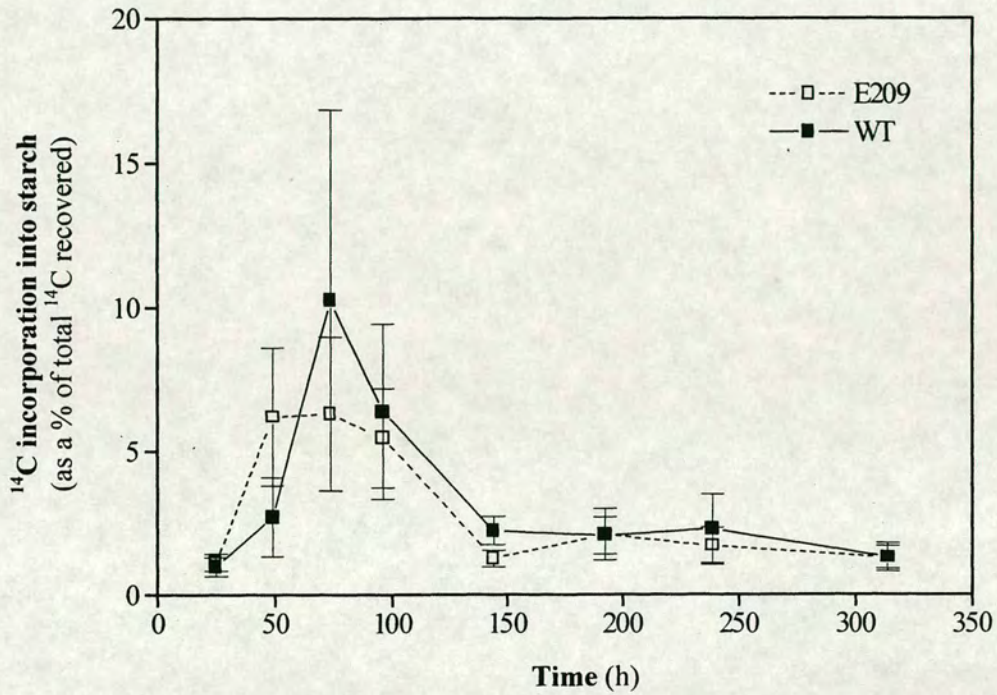


Figure 5. The incorporation of $[\text{U}^{14}\text{C}]$ sucrose into starch after a 3 h pulse into wild type and E209 mature tubers.

Tubers were harvested from plants which had been grown in a heated greenhouse August - December 1999. $[\text{U}^{14}\text{C}]$ sucrose was delivered to mature tubers which had been stored at 4°C for 14 weeks through a bore hole midway between the apical and basal ends of each tuber. The radiolabel was left in the tuber for 3 h, then thoroughly washed out and replaced with cold sucrose for the chase. Tuber tissue was sampled as described in chapter 2. The incorporation of label into starch was expressed as a percentage of the total label recovered. Each value is the mean of three individual tubers. Error bars indicate s.e.m..

4.3 Discussion

The starch contents of leaves with lowered D-enzyme activity were analysed after exposure to light and darkness. The quantity of leaf starch at the start of the dark period was found to be higher in leaf tissue from E211 than in wild type. A similar phenotype has also been observed in the leaves of the *A. thaliana* D-enzyme mutant *dpe1-1* (Critchley *et al.*, 2000). However, only small differences were detected in the rates of starch synthesis and degradation between leaf tissue from E211 and wild type plants. It is interesting to note that although the differences in rates of starch breakdown were small, the leaf starch content of E211 was greater than that of wild type at each time point. Lack of D-enzyme in E211 did appear to result in a small reduction in the rate of starch degradation in the early part of the dark period relative to wild type. The cumulative effect of a small reduction in the rate of starch degradation over several diurnal cycles could result in the greater quantities of leaf starch which were observed in E211 at the start of the dark period. This proposal is consistent with the effect of lack of D-enzyme activity on leaf starch breakdown in leaves of *dpe1-1* in which the mobilisation of leaf starch was also found to be reduced in darkness (Critchley *et al.*, 2000). After 17 h in light the rate of starch synthesis in E211 was slightly lower than that of wild type plants, although after 17 h the rate of leaf starch synthesis in E211 was greater than wild type. The greater quantity of leaf starch in E211 relative to wild type plants at the start of the dark period is not consistent with a role for D-enzyme in leaf starch synthesis. No evidence was found to support a role for D-enzyme in leaf starch synthesis in *dpe1-1* (Critchley *et al.*, 2000). The reduction in leaf starch breakdown in E211 is small. However, when viewed in the context of the altered starch metabolism of *dpe1-1* it appears likely that lack of D-enzyme in E211 results in a reduction in the rate of starch breakdown in the leaves of this plant during darkness. The reduction in leaf starch degradation in *dpe1-1* was suggested to be due to either an indirect effect of an accumulation of MOS which occurs in the leaves of *dpe1-1* in darkness or due to a direct role for D-enzyme in the degradation of starch molecules (Critchley *et al.*, 2000). No evidence was presented in this chapter to indicate an exact role for D-enzyme in starch degradation in potato leaves.

Leaf starch content was also analysed by staining with iodine. Based on the staining intensity of leaves from E211 and wild type after 12 h of darkness, it might have been expected that the actual starch content of E211 relative to wild type when determined biochemically would

have been greater than the values which were actually observed. One reason for this could be that the proportion of amylose in the leaf starch of plants lacking D-enzyme was greater than in wild type. Amylose stains dark blue in the presence of iodine whereas amylopectin stains a red-brown colour. This difference relates to the higher affinity of amylose for iodine compared to that of amylopectin. Therefore the staining technique is likely to misrepresent the quantity of starch if amylose : amylopectin ratios are altered. The amylose contents of plants at three stages of development were investigated. The proportion of amylose in leaf starch from flowering plants was significantly higher in plants lacking D-enzyme than in wild type. The leaf starch from pre-flowering *A. thaliana* D-enzyme mutant, *dpe1-1*, plants was also found to have a higher amylose content than wild type (Critchley, *et al.*, 2000). However, the proportion of amylose in leaf starch from developing potato plants was not affected by lack of D-enzyme. It is possible that the different amylose contents between the flowering and developing plants could have been due to differences in the developmental stages of these plants rather than to the effect of lack of D-enzyme. In many species the amylose content of leaves has been shown to vary with development. For example, increases in apparent amylose content have been correlated with leaf maturity in cotton and tobacco (Chang, 1979; Matheson, 1996). In senescing leaves, leaf amylose content is reported to remain stable in tobacco (Abbot and Matheson, 1972) but was found to fall in cotton leaves (Chang, 1980). If differences in the developmental stages of plants lacking D-enzyme and wild type affect the amylose content this could be particularly relevant during flowering. The growth of plants lacking D-enzyme is slower than wild type for the duration of the growth period (Takaha *et al.*, 1998c; chapter 3). Lack of D-enzyme also delays the flowering of these plants compared to wild type (Takaha, 1996). During flowering there are substantial changes in the sink-source relations of a plant (Wardlaw, 1990). It should be mentioned that fluctuations in growth conditions may have affected absolute amylose levels and could account for the range of amylose contents that were observed between the three experiments. Leaf amylose contents have been reported to fluctuate diurnally in cotton and tobacco leaves and also in response to length of illumination in bean chloroplasts (Kovacs and Hill, 1974; Chang, 1979; Matheson, 1996). No strong evidence was found to suggest that the proportion of amylose in potato leaf starch was altered due to lack of D-enzyme. However, these results are important for showing the extent to which amylose levels fluctuate during plant growth.

Plant metabolism is highly complex and tightly regulated, making it extremely difficult to interpret the effects of perturbations on metabolism and attribute them to specific causes (Herbers and Sonnewald, 1996). Based on other reports it is conceivable that a lack of D-enzyme in leaves could potentially have other non-direct effects on plant metabolism. For instance, in potato a reduction in leaf sucrose export by expression of yeast invertase was reported to lower the photosynthetic capacity of these plants through changes on gene expression (Heineke *et al.*, 1992; Krapp *et al.*, 1993). A reduction in photosynthetic capacity could contribute to the reduction in growth of low D-enzyme expressing plants. This could also explain the effect of irradiance level on the extent of the reduction on tuber yield (chapter 3). In addition, it is possible that alterations in carbohydrate metabolism in the light could partially compensate for lack of D-enzyme. This was found to be the case for *dpe1-1* where rates of starch synthesis were reduced during the diurnal cycle (Critchley *et al.*, 2000). Diurnal carbohydrate metabolism is reported to be highly flexible in potato. For example, increases in leaf starch due to antisense repression of the triose-P translocator was found to result in increased carbon export in the dark (Riesmeier *et al.*, 1993; Hausler *et al.*, 1998). Conversely, a reduction in leaf starch due to leaf-specific antisense repression of AGPase was found to result in a greater mobilisation of carbon during the day (Leidreiter *et al.*, 1995). In other reports the amount of starch present at the start of the dark period was found to influence the rate of starch breakdown in the dark (Zrenner *et al.*, 1996; Sweetlove and Hill, 2000). Further experiments are required to ascertain the relevance of these reports to the lowering of D-enzyme activity in potato.

No differences were detected in rates of starch synthesis or turnover in tubers which lack D-enzyme. Both developing and sprouting tubers exhibited patterns of concomitant synthesis and degradation thought to be characteristic of starch cycling and in agreement with other studies (Geigenberger *et al.*, 1994; Trethewey *et al.*, 1999; Sweetlove *et al.*, 1996b). Despite high levels of variation the amount and the pattern of incorporation of label into starch was surprisingly similar between tubers with lowered D-enzyme activity and wild type. If small differences did exist in the metabolism of these tubers it is likely they would have been masked by the high variation which was observed between individual tubers. Sprouting tubers exhibit a net degradation of starch so the relatively high incorporation of label into starch in sprouting tubers and the failure to detect differences between sprouting wild type and non-sprouting E209 tubers is not readily explained. If, in the sprouting tubers, starch was

not metabolised uniformly throughout the tuber then the method of sampling tissue from 1 mm bore holes may have precluded local differences in metabolism from being represented in the analysis. However, there are other reports which describe anomalous results from labelling experiments. For example, in potato engineered to express a tuber-specific yeast invertase the flux of label into starch was shown not to correlate with the observed reduction in tuber starch content (Trethewey *et al.*, 1999). In the same report the measurement of flux of label into starch varied markedly between different experiments. The interpretation of labelling experiments can be extremely complex. The failure to detect differences between the starch metabolism of wild type and E209 tubers does not necessarily mean that differences do not exist between these tubers.

The analysis of leaf and tuber starch metabolism discussed above is pertinent to the reduction in tuber yield associated with a lack of D-enzyme which is described in chapter 3. The influence of sink and source metabolism on resource allocation has been the subject of a long-standing debate (for review see Wardlaw, 1990). Some authors have suggested that carbon flux is sink-limited (e.g. Geiger, 1976). More recently studies of carbon flux between 'blocks' of sink and source reactions have provided strong evidence that up to 90 % of the control of flux is accounted for by source metabolism (Sweetlove *et al.*, 1998; Sweetlove and Hill, 2000). Therefore, it is possible that a reduction in the capacity of E211 leaves to degrade starch could account for the observed reduction in tuber yield associated with lack of D-enzyme. An alternative explanation put forward in chapter 3 to explain the reduction in tuber yield was that it could be a result of a disruption of tuber starch synthesis. However, this is unlikely due to the normal tuber induction, tuber development and tuber starch of plants lacking D-enzyme (Takaha *et al.*, 1998c; chapter 3) and the limited influence of source tissue on whole plant metabolism which was described above. In other reports leaf metabolism has been shown to affect sink metabolism. For example, a reduction in leaf apoplastic sucrose by expression of yeast invertase was reported to lead to reductions in sink growth rates in potato (Heineke *et al.*, 1992). Conversely, increased light and CO₂ have been reported to increase tuber yield (Yandell *et al.*, 1988).

Evidence was presented in this chapter which indicated that lack of D-enzyme in line E211 resulted in a small reduction in leaf starch degradation in darkness. These results are in agreement with those from the *A. thaliana* mutant, *dpe1-1*, and suggest that lack of D-

enzyme results in a reduction in the capacity of these plants to degrade starch during darkness. No evidence was presented in this chapter to indicate an exact role for D-enzyme in leaf starch degradation in potato. From the experiments on leaf tissue, no evidence was found to support the proposal by Colleoni *et al.* (1999b) that D-enzyme is involved in starch synthesis. There were no detectable differences in the rates of starch synthesis and turnover between tubers which lacked D-enzyme and wild type. This was surprising as lack of D-enzyme results in a delay of tuber sprouting and this is thought to be caused by a disruption to the mobilisation of starch (Takaha *et al.*, 1998c). The following chapter describes an alternative approach to investigate the function of D-enzyme in tubers.

Chapter 5. The Production of Transgenic *Solanum tuberosum* L. cv. Prairie Plants with Altered D-enzyme Activity

5.1 Introduction

The work described in chapter 4 provided evidence to support a role for D-enzyme in starch degradation in potato leaves. Experiments on tubers failed to detect any differences in the rates of starch synthesis or starch turnover in tubers with lowered D-enzyme activity compared to controls. However, it is known that lack of D-enzyme does effect tuber metabolism as indicated by the reduction in fresh weight yield, delay in tuber sprouting and delay in sugar accumulation in these tubers during storage (Takaha *et al.*, 1998c; chapter 3). It is possible that the experimental system employed in chapter 4 was not sensitive enough to detect differences in tuber metabolism. To address this it was decided to lower D-enzyme activity in potato plants which exhibit increased rates of starch synthesis and turnover. In this alternative system potential differences in starch metabolism due to lack of D-enzyme may be more readily resolved. Potato plants cv. Prairie engineered to express the *glgC-16* gene from *E. coli* were reported to exhibit increased rates of starch synthesis but did not accumulate starch due to increased rates of starch turnover (Sweetlove *et al.*, 1996a, b). The work in this chapter describes the production and screening of transgenic potato plants cv. Prairie and Prairie *glgC-16* with lowered D-enzyme activity.

Lowering D-enzyme activity in Prairie *glgC-16* plants is a re-transformation process. Prairie *glgC-16* plants contain the *nptII* gene which provides resistance to kanamycin. A second selectable marker was therefore required for the re-transformation of these plants. It was decided to use a sulphonamide resistance gene *sulI*. Sulphonamides are anti-bacterial compounds that inhibit dihydropteroate synthase (DHPS), an enzyme of the folic acid synthesis pathway. DHPS catalyses a rate limiting step in folic acid synthesis in both plants and bacteria (Cossins, 1980). *SulI* encodes a mutant form of DHPS that is insensitive to inhibition by sulfonamides and can complement the inhibited plant enzyme (Wise *et al.*, 1975; Gurineau *et al.*, 1990). The plasmid constructs used for plant transformation in this

study contained both *nptII* and *sulI*. To compare the efficiencies of the two selection agents Prairie and Prairie *glgC-16* lines were selected on kanamycin and sulphadiazine respectively.

Prairie and Prairie *glgC-16* plants were transformed with plasmid constructs containing sense or antisense orientation potato D-enzyme cDNA. The introduction of homologous sense cDNA can result in over expression or co-suppression of the target gene. Over expression is more likely to be achieved by the use of a heterologous gene (Worell *et al.*, 1991; Gaultier *et al.*, 1993). Only D-enzyme cDNA from potato was available at the start of this project. Transgenic plants were produced by *Agrobacterium tumefaciens* mediated transformation of leaf discs. Co-cultivated leaf discs were first induced to form calli, then shoots and then roots by maintenance on successive sets of specialised media. Transformed shoots were transferred to microtuber induction medium. Microtuber protein was used to screen transformants by western blotting. The carbohydrate metabolism of microtubers does not differ markedly from soil-grown tubers despite the different sources of sucrose (e.g. Hovenkamp and Hermelink, 1988; Veramendi, *et al.*, 1999). Microtubers have the advantage of being able to be generated several magnitudes faster than soil-grown tubers. Lastly, soil-grown tubers from regenerated plants were screened for D-enzyme activity to enable the selection of lines for further analysis.

5.2 Results

5.2.1 Construction of plant transformation plasmids

Two recombinant plasmids for use in plant transformation were produced. p10025 was designed to induce over expression or co-suppression of D-enzyme. p10026 was designed to lower D-enzyme expression through antisense technology. In each plasmid D-enzyme cDNA was linked to a constitutive CaMV 35 S promoter. p10025 and p10026 contained D-enzyme cDNA in sense or antisense orientation respectively and *nptII* and *sulI* as selectable markers (figure 1). The constructs were based on the binary vector pJIT119 (figure 2). pJIT119 was linearised by digesting with *XbaI*. Potato D-enzyme cDNA from pDPE102N (figure 3) was amplified by PCR to include *SpeI* endonuclease restriction enzyme recognition sites at 5' and 3' ends. The D-enzyme cDNA was cloned into pJIT119 in both directions.

5.2.2 Characterisation of plasmid constructs

5.2.2.1 Restriction endonuclease digest and PCR analysis of plasmid DNA from electroporated *E. coli*

p10025 and p10026 were electroporated into *E. coli*. To check the integrity of the constructs DNA isolated from *E. coli* was characterised by endonuclease restriction digest analysis. The sizes of the resulting DNA fragments were consistent with the presence of D-enzyme cDNA in sense orientation in p10025 and in antisense orientation in p10026 (data not shown). The plasmid constructs were also analysed by PCR. DNA from *E. coli* electroporated with p10025 and p10026 was amplified using primers homologous to 5' and 3' ends of D-enzyme cDNA. The PCR products from these reactions were of identical size to D-enzyme cDNA amplified from control plasmids (data not shown). In addition, PCR with primers homologous to CaMV 35 S promoter and CaMV poly A terminator sequences indicated sense orientation of the PCR product in p10025 and antisense orientation in p10026 (data not shown).

5.2.2.2 Sequence analysis of plant transformation constructs

The promoter and terminator border sections of p10025 and p10026 were sequenced by Advanced Technologies (Cambridge) Ltd. It was confirmed that D-enzyme cDNA was present in sense orientation in p10025 and in antisense orientation in p10026.

5.2.2.3 Restriction endonuclease digest analysis of DNA from electroporated *A. tumefaciens*

Prior to use in plant transformation the constructs were electroporated into *A. tumefaciens*. To check the integrity of the constructs DNA isolated from *A. tumefaciens* was characterised by endonuclease restriction digest analysis. The sizes of the resulting DNA fragments were identical to those observed from the plasmids expressed in *E. coli*, see section 5.2.2.1 (data not shown).

5.2.3 Production of transgenic plants

A. tumefaciens mediated transformation was used to transfer the region between the right and left T-DNA borders of p10025 and p10026 into potato leaf discs. 600 Prairie and 1300 Prairie *glgC-16* leaf discs were co-cultivated in *A. tumefaciens* which had been electroporated with p10025 or p10026. The discs were plated on successive sets of media to select transformants and promote shoot and root induction. Transformed controls were co-cultivated with *A. tumefaciens* containing pJIT119. Prairie and Prairie *glgC-16* transformant lines were selected on kanamycin and sulphadiazine respectively. The presence of the transgene in regenerated shoots from leaf discs which had been co-cultivated with p10025 and p10026 was checked by PCR. The presence of the transgene in transformed control plants was checked by assaying β -glucuronidase activity. The efficiency of shoot regeneration and the proportion of regenerated shoots which were transgenic is shown in table 1. The number of regenerated shoots was highly variable. Generally a lower proportion of regenerated shoots were obtained from Prairie *glgC-16* lines and from co-cultivations with pJIT119. There was no difference in the proportion of regenerated shoots which were transgenic between the different plasmid constructs and plant lines.

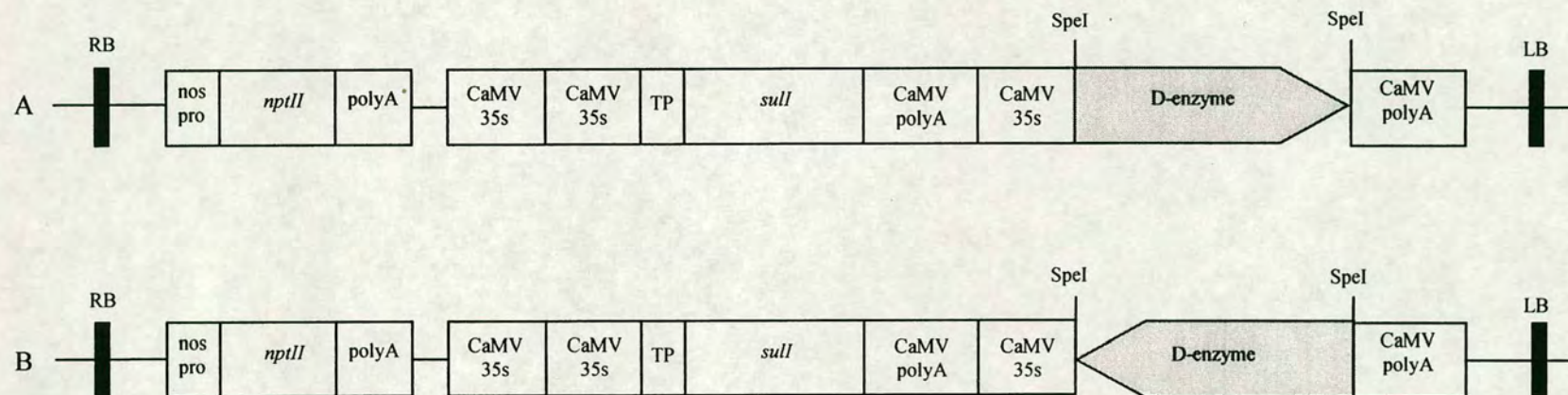


Figure 1. The right to left border sections of plant transformation plasmids p10025 and p10026.

p10025 and p10026 were assembled using the methods described in chapter 2. The cloning strategy involved amplifying D-enzyme cDNA from pDPE102N, to include *SpeI* endonuclease restriction enzyme recognition sites at 5' and 3' ends. The D-enzyme cDNA was ligated into a binary plasmid, pJIT119, which had been linearised by digestion with *XbaI* (*SpeI* and *XbaI* are compatible sites for ligation). A) sense orientation D-enzyme cDNA - p10025 and B) antisense orientation D-enzyme cDNA - p10026.

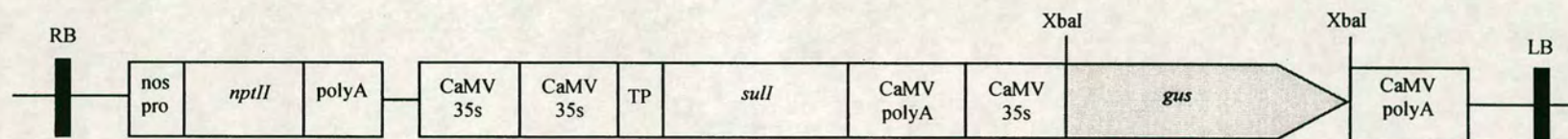


Figure 2. The right to left border section of pJIT119.

pJIT119 was made by Gurineau *et al.* (1990). The construct was produced by inserting a transit peptide, TP- *sull* coding sequence between the right and left T-DNA borders of pBIN19 (Bevan, 1984). *sull* = sulphonamide resistance gene, TP = pea ribulose biphosphate carboxylase / oxygenase small subunit TP, CaMV 35s = CaMV 35 S promoter (the promoters driving *sull* contain a duplicated enhancer region), CaMV polyA = CaMV polyadenylation signal, *nptII* = kanamycin resistance gene, *nos* = nopaline synthetase promoter, *gus* = β -glucuronidase gene.

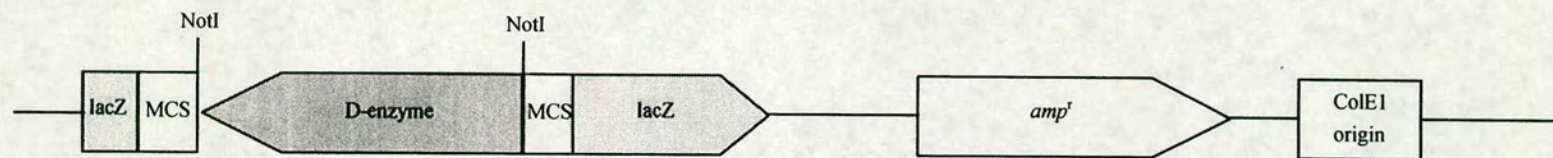


Figure 3. The right to left border section of pDPE102N.

pDPE102N was constructed by Takaha *et al.* (1993). pDPE102N was assembled by cloning D-enzyme cDNA into the NotI site of pBluescript II SK+. LacZ = β -galactosidase gene, MCS = multiple cloning site, *amp^r* = Ampicillin-resistance gene, ColE1 origin = plasmid origin of replication.

Plasmid	Prairie		Prairie <i>glgC-16</i>	
	Number of shoots regenerated in tissue culture (as a percentage of total number of co-cultivations)	Number of regenerated shoots which are transgenic (as a percentage of total number of regenerated shoots)	Number of shoots regenerated in tissue culture (as a percentage of total number of co-cultivations)	Number of regenerated shoots which are transgenic (as a percentage of total number of regenerated shoots)
p10025	68	67	8	73
p10026	28	70	17	71
pJIT119	15	60	1	100

Table 1. The efficiencies of shoot regeneration and transformation in Prairie and Prairie *glgC-16* leaf discs co-cultivated with p10025, p10026 and pJIT119.

Leaf discs from 4-5 week old Prairie or Prairie *glgC-16* plants were co-cultivated with *A. tumefaciens* containing p10025, p10026 or pJIT119. The leaf discs were maintained for several weeks on successive sets of media designed to select transformed lines and promote shoot and root growth. The methods of plant transformation and *in vitro* cultivation are described in chapter 2. The number of leaf discs which produced shoots is expressed as a percentage of the number of discs that were co-cultivated for each line. The presence of D-enzyme cDNA in the transformed shoots was analysed by PCR. The number of regenerated shoots which contained D-enzyme cDNA is expressed as a percentage of the number of regenerated shoots. For pJIT119 controls confirmation that lines were transgenic was established through the assay of β -glucuronidase activity of leaf tissue.

5.2.4 Protein screening of regenerated plants

The level of D-enzyme expression in protein from microtubers (figure 4) which had been grown from regenerated transgenic shoots was investigated by western blotting. Control plants consisted of Prairie and Prairie *glgC-16* lines which had been co-cultivated with pJIT119 and were shown to express β -glucuronidase activity (section 5.2.3). These lines are referred to as Prairie GUS-controls and Prairie *glgC-16* GUS-controls from this point onward. Protein from *in vitro* grown microtubers from Prairie transformants (figure 5) and Prairie *glgC-16* transformants (figure 6) was probed with D-enzyme antisera. None of the p10025 (sense) transformants from either plant line exhibited protein levels which differed from the respective GUS-control tubers. For the p10026 (antisense) lines 37 Prairie transformants were screened. Of these, 8 showed protein levels lower than Prairie GUS-control tubers. 59 Prairie *glgC-16* transformants were screened. Of these, 4 showed protein levels lower than Prairie *glgC-16* GUS control tubers.

5.2.5 D-enzyme activity screen

The plant lines identified as expressing lowered levels of D-enzyme protein (section 5.2.4) were weaned from *in vitro* growth media onto soil. Developing tubers were sampled from 14 week-old plants and assayed for D-enzyme activity (figure 7). Of the Prairie lines, 3 independent transformant lines exhibited D-enzyme activities which were significantly lower than Prairie GUS-control tubers. Lines 1, 41 and 44 exhibited 6.3 %, 12.4 % and 17.3 % of Prairie GUS-control D-enzyme activity respectively. Of the Prairie *glgC-16* lines, 2 independent transformant lines exhibited D-enzyme activities which were significantly lower than Prairie *glgC-16* GUS-control tubers. Lines 59 and 101 exhibited 15.2 % and 51.2 % of Prairie *glgC-16* GUS-control D-enzyme activity respectively.



Figure 4. Potato microtubers on nodal cuttings from regenerated shoots.

Microtubers were induced from nodal cuttings of regenerated shoots as described in chapter 2.

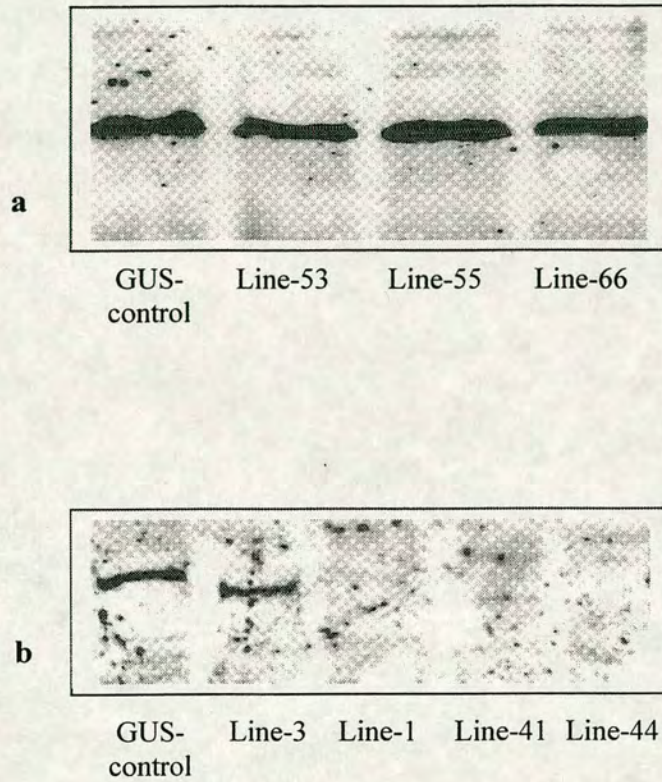


Figure 5. The levels of D-enzyme protein expression in Prairie transgenic lines transformed with a) p10025 (sense) and b) p10026 (antisense).

Leaf discs from 4-5 week old Prairie plants were co-cultivated with *A. tumefaciens* containing p10025, p10026 or pJIT119. The leaf discs were cultured *in vitro* and induced to produce shoots and roots. The level of D-enzyme protein expression in microtubers produced from nodal cuttings of regenerated shoots was investigated by western blotting. 20 µg protein was probed with potato D-enzyme antisera according to the methods described in chapter 2. A sample of the independent transformants which were screened is shown for each plant line.

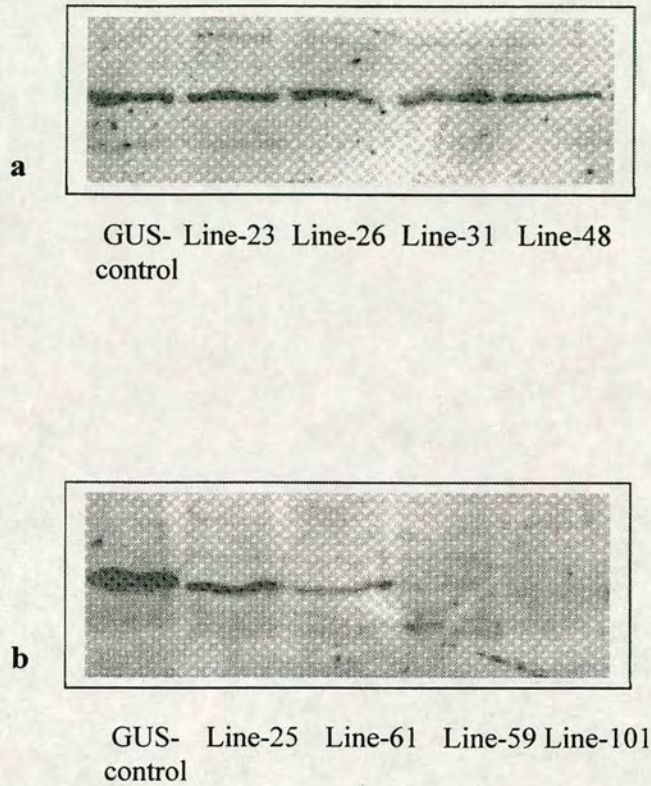


Figure 6. The levels of D-enzyme protein expression in Prairie *glgC-16* transgenic lines transformed with a) p10025 (sense) and b) p10026 (antisense).

Leaf discs from 4-5 week old Prairie *glgC-16* plants were co-cultivated with *A. tumefaciens* containing p10025, p10026 or pJIT119. The leaf discs were cultured *in vitro* and induced to produce shoots and roots. The level of D-enzyme protein expression in microtubers produced from nodal cuttings of regenerated shoots was investigated by western blotting. 20 μ g protein was probed with potato D-enzyme antisera according to the methods described in chapter 2. A sample of the independent transformants which were screened is shown for each plant line.

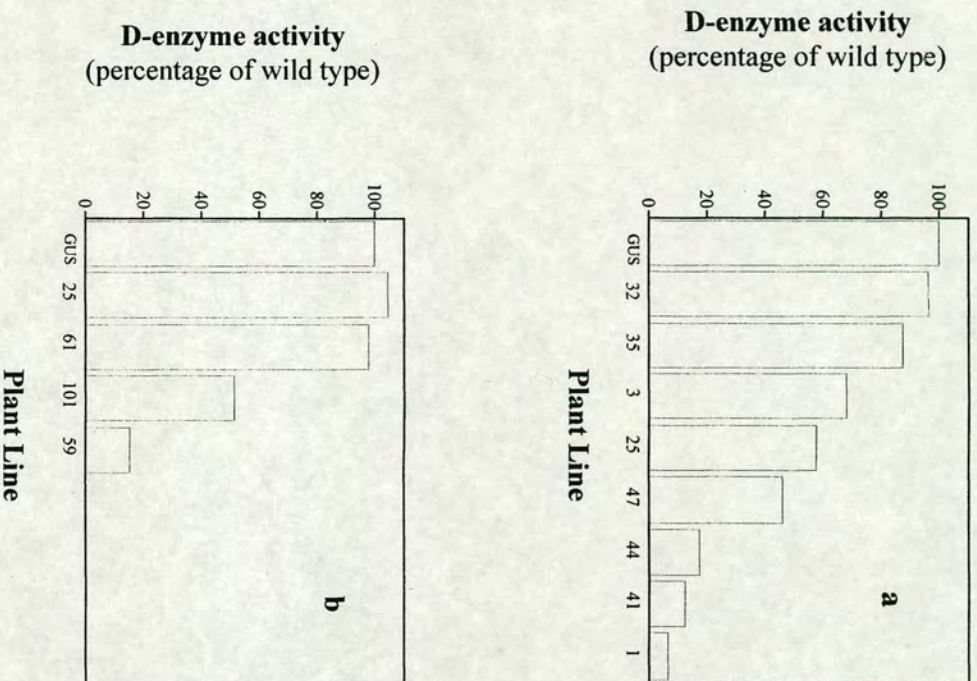


Figure 7. The levels of D-enzyme activity in tubers from transgenic a) *Prairie* and b) *Prairie glgC-16* plants.

In vitro grown plantlets from a) *Prairie* and b) *Prairie glgC-16* transformants were weaned on to soil. Developing tubers were harvested from 14 week-old plants and assayed for D-enzyme activity as described in chapter 2. D-enzyme activities are expressed as a percentage of wild type activity.

5.3 Discussion

This chapter describes the production of transgenic plants from *Prairie* and *Prairie glgC-16* potato plants with lowered D-enzyme activity. *Prairie* and *Prairie glgC-16* leaf discs were co-cultivated with the plasmid constructs p10025 (sense) and p10026 (antisense) and shoots were regenerated from the leaf discs. From co-cultivations with p10026, approximately 70 % of the regenerated shoots from both *Prairie* and *Prairie glgC-16* leaf discs were found to contain D-enzyme cDNA. Only a small proportion of these transformants exhibited lowered D-enzyme activity. The extent of the reduction in D-enzyme activity in these lines was disappointing. For both plant lines, 5 out of a total of 1300 individual co-cultivations with p10026 yielded plants with D-enzyme activities between 6 to 50 % of wild type. None of the co-cultivations with p10025 yielded plants with altered D-enzyme activity.

It is characteristic for transformants from antisense experiments to exhibit large and unpredictable variation in gene expression (e.g. Peach *et al.*, 1991; Hobbs *et al.*, 1993). There are multiple reasons put forward to explain this. Unfavourable transgene insertion as a result of deletion of nucleotides at recombining ends, position effects, rearrangement of the target gene and the specificity of the antisense sequence for RNA can all abolish transgene expression (Stam *et al.*, 1997; Kohli *et al.*, 1998, 1999). More than one T-DNA copy may be needed to silence all copies of the target gene in polyploid plants such as potato (Wolters *et al.*, 1998). In addition, if multiple alleles have different homologies to the inserted gene this may result in different levels of expression (van Eck, 1994).

The efficiency of shoot regeneration from *Prairie glgC-16* lines selected on sulphadiazine was lower than that of the *Prairie* lines which were selected on kanamycin. This indicates that sulphadiazine used at the concentration employed in this study had a negative effect on the ability of leaf discs to regenerate shoots. This finding conflicts with a report which describes equal efficiencies of sulphadiazine and kanamycin at enabling shoot regeneration (Wallis *et al.*, 1986). One reason for this discrepancy could be that different cultivars were used in these studies. In this work *Prairie* was used whereas Wallis *et al.* (1986) used Russet Burbank. Different cultivars are known to exhibit variation both in transformation efficiency and in morphogenesis in tissue culture (Dale and Hampson, 1995). Another reason could be differences in the expression of *nptII* and *sulI* as the two genes are driven by different promoters in p10026. The number of regenerated shoots which were transgenic was similar

for each line indicating that sulphadiazine was as efficient as kanamycin at selecting transformed lines.

The levels of D-enzyme activity in tubers from Prairie lines 1, 41 and 44 and in tubers from Prairie *glgC-16* lines 59 and 101 were significantly reduced. For the analysis of transgenic plants it is usual practice to study three independent transformants. This should prevent any potential artefacts induced by somoclonal variation from being treated as authentic phenotypes. In the case of the Prairie *glgC-16* lines this is not possible as only two transformants with lowered D-enzyme activity were isolated. The extent of the reduction of D-enzyme activity in the Prairie and Prairie *glgC-16* lines ranged from 6 - 50 % of the GUS-control lines. The reduction of D-enzyme activity in May Queen tubers (Takaha, 1996; chapter 3) was greater than that achieved with the Prairie and Prairie *glgC-16* lines. Several May Queen lines exhibited activities below 3 % of wild type. Despite this it was decided that the reductions in D-enzyme activity were low enough to warrant an investigation of the phenotypes of these plants. This work is the subject of the following chapter.

Chapter 6. Analysis of *Solanum tuberosum* L. cv. Prairie with Lowered D-enzyme Activity

6.1 Introduction

To date the sole phenotype of potato tubers which lack D-enzyme is a delay in sprouting after prolonged storage (Takaha, 1996; chapters 3 and 4). From our knowledge of tuber physiology it is thought that this is due to a disruption of starch mobilisation - the mechanism required to fuel heterotrophic sprout growth (Burton, 1989). If this is the case then it should be possible to detect changes in starch mobilisation in tubers lacking D-enzyme when a suitable experimental system is employed. Potato plants cv. *Prairie* have been engineered previously to express the *glgC-16* gene from *E. coli*. Tubers from these plants were reported to exhibit increased rates of starch synthesis, but did not accumulate starch due to increased rates of starch turnover (Sweetlove *et al.*, 1996a, b). If lack of D-enzyme does result in a reduction in tuber starch mobilisation the effect of this may be more readily resolved in *Prairie glgC-16* tubers than in the *May Queen* tubers, which were studied in chapter 4. In the previous chapter D-enzyme activity was lowered in *Prairie* and *Prairie glgC-16* plants. The aim of the work presented in this chapter was to characterise *Prairie* and *Prairie glgC-16* plants with lowered D-enzyme activity.

The *glgC* gene encodes *E. coli* AGPase, a homotetrameric enzyme which is activated by fructose 1,6-bisphosphate and inhibited by AMP and Pi (Priess *et al.*, 1966; Okita *et al.*, 1981). The *glgC-16* gene encodes a mutant form of the enzyme which exhibits a reduction in response to allosteric regulation (Cattaneo *et al.*, 1969). Potatoes expressing a plastidial-targeted *glgC-16* protein under the control of a patatin promoter have been characterised extensively in the cultivar *Prairie* (Sweetlove *et al.*, 1996 a, b). In these plants AGPase activity was reported to be increased by up to 400% but tuber starch content was unaltered. [^{14}C]sucrose feeding experiments indicated that flux into starch was increased roughly in proportion to the increase in AGPase activity but the tubers also had an increased capacity to degrade starch. In chapter 4, potato tubers with lowered D-enzyme activity from the cultivar *May Queen* were analysed by similar [^{14}C]sucrose feeding experiments. No appreciable differences were detected in the metabolism of ^{14}C into or out of starch between

tubers with wild type and lowered levels of D-enzyme activity. Only low rates of starch turnover could be detected in tubers from these plants. In contrast to this, the high rates of starch turnover in *glgC-16* tubers may provide a more suitable system for detecting potential differences in tuber starch degradation. In addition, the increased rates of starch synthesis in *glgC-16* tubers may provide a system in which potential perturbations to starch synthesis could be detected. It was suggested previously that the reduction in tuber yield of plants which lack D-enzyme could either be due to a reduction in carbon export from leaves or due to a reduction in tuber starch synthesis (chapter 4). At present there is no evidence to support a role for D-enzyme in tuber starch synthesis but such a role cannot be ruled out. Analysis of starch synthesis in these tubers was therefore also warranted.

Initially, potential changes in non-target enzymes involved in starch metabolism were investigated in *Prairie* and *Prairie glgC-16* tubers with lowered D-enzyme activity. Experiments were then directed towards investigating features which have been reported to be altered in *May Queen* potato plants with lowered D-enzyme activity (Takaha, 1996; chapters 3 and 4). Plant growth, tuber yield, tuber sprouting and leaf starch metabolism were analysed. Lastly, labelling experiments were employed to study the effect of lowered D-enzyme activity on tuber starch metabolism. All of the experiments described in this chapter were performed on plants which were grown in a heated greenhouse January - July 2000.

6.2 Results

6.2.1 Activities of starch metabolic enzymes in Prairie and Prairie *glgC-16* tubers with lowered D-enzyme activity

The possibility that the introduction of D-enzyme cDNA may have affected non-target enzymes was investigated by assaying other starch metabolic enzymes in these plants. Assays were performed on developing tubers harvested from 12 week-old plants. The maximum catalytic activities of enzymes from Prairie lines 1, 41 and 44, Prairie GUS-control lines, Prairie *glgC-16* lines 59 and 101 and Prairie *glgC-16* GUS-control lines are shown in table 1. Within the Prairie and Prairie *glgC-16* plant lines, D-enzyme activity was significantly different between the transgenic lines and respective controls (when analysed by one-way ANOVA with a confidence limit of 99.5 %). AGPase activity was significantly different between Prairie and Prairie *glgC-16* plant lines but there was no significant difference in AGPase activity between the transgenic lines and controls within each of the plant lines (when analysed by one-way ANOVA with a confidence limit of 99.5 %). β -amylase and soluble starch synthase activities tended to be higher in Prairie *glgC-16* plants than in Prairie plants but this difference was not significant (when analysed by one-way ANOVA with a confidence limit of 95 %).

6.2.2 Growth of Prairie and Prairie *glgC-16* plants with lowered D-enzyme activity

Plant growth was followed by measuring plant height at regular intervals throughout the growth period (figure 1). There were no significant differences between the height of plants with lowered D-enzyme activity and controls, both within each plant line and between the Prairie and Prairie *glgC-16* plant lines at each time point tested (when analysed by one and two-way ANOVA with a 95 % confidence limit).

6.2.3 Tuber yield of Prairie and Prairie *glgC-16* plants with lowered D-enzyme activity

The yield of tubers harvested from Prairie and Prairie *glgC-16* plants is shown in table 2. Values for specific gravity were obtained by weighing tubers in water and comparing this to fresh weight. Specific gravity values were used to calculate the proportion of tuber dry matter and starch according to von Scheele *et al.* (1937).

Enzyme	Activity (nmol min ⁻¹ g ⁻¹ Fresh Weight)						
	PR-GUS	PR-1	PR-41	PR--44	PT-GUS	PT-59	PT-101
D-enzyme	2363.1 ± 251.9	247.2 ± 29.9	342.7 ± 41.9	284.9 ± 55.4	2179.9 ± 302.8	313.7 ± 31.9	1125.5 ± 135.8
AGPase	159.4 ± 30.1	130.8 ± 19.7	176.9 ± 34.4	130.7 ± 22.4	733.1 ± 96.6*	645.3 ± 58.1*	765.9 ± 63.0*
Starch synthase	75.2 ± 21.1	68.3 ± 16.5	77.7 ± 17.8	69.1 ± 19.5	93.5 ± 32.8	76.1 ± 21.5	83.7 ± 26.6
Starch phosphorylase	279.0 ± 38.0	222.4 ± 25.6	310.1 ± 33.5	227.9 ± 49.2	223.5 ± 65.5	281.1 ± 42.7	233.8 ± 23.2
α-amylase	88.4 ± 17.5	97.1 ± 10.2	90.0 ± 10.3	82.8 ± 9.61	90.0 ± 4.9	103.5 ± 8.1	92.3 ± 5.2
β-amylase	6.8 ± 2.3	7.3 ± 3.4	7.4 ± 3.1	5.9 ± 2.1	16.9 ± 5.8	18.0 ± 6.3	20.2 ± 4.4

Table 1. The activities of D-enzyme and other starch metabolic enzymes in Prairie and Prairie *glgC-16* potato tubers with lowered D-enzyme activity and GUS-controls.

In vitro grown plantlets were weaned onto soil and grown in a heated greenhouse at 20 °C, 16 h day length and at least 300 μmol m⁻² sec⁻¹ irradiance. Tubers were harvested from 12 week-old plants and were assayed for enzyme activities as described in chapter 2. All assays had been previously optimised (section 3.2.1 and appendix). PR and PT indicate Prairie and Prairie *glgC-16* lines respectively. The values are the means of three individual tubers ± the s.e.m.. Values in bold are significantly different from their respective control plants (analysed by one-way ANOVA with a 99.5 % confidence limit). * indicates values from Prairie *glgC-16* plants which are significantly different to Prairie plants (analysed by two-way ANOVA with a 95 % confidence limit).

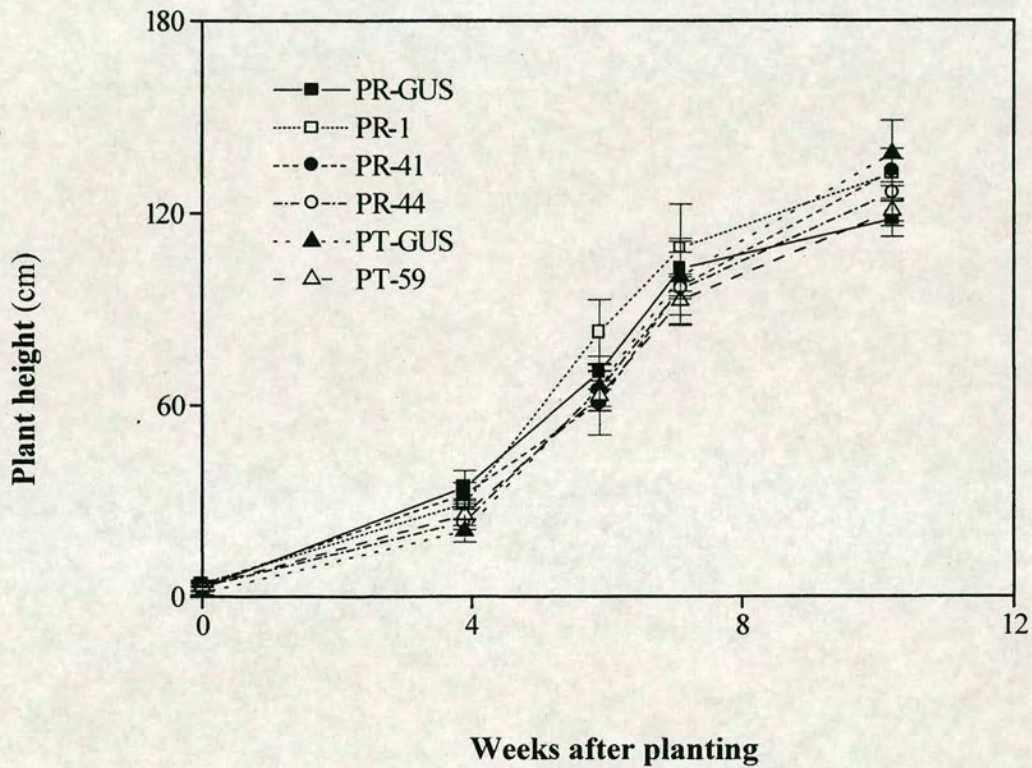


Figure 1. The growth of Prairie and Prairie *glgC-16* lines with lowered D-enzyme activity and GUS-controls.

Plants were grown from tubers which had been kept at 12 °C until they produced sprouts. All the sprouts were removed except the strongest one. Tubers were planted on the same day in identical pots of 25 cm diameter and were grown in random positions in an unheated greenhouse between April - September. PR and PT indicate Prairie and Prairie *glgC-16* lines respectively. The values for each time point are the mean of three individual plants. Error bars indicate s.e.m..

There were no significant differences between the fresh weight yields, specific gravity, dry matter and starch contents of tubers with lowered D-enzyme activity and controls, both within each plant line and between the two plant lines (when analysed by two-way ANOVA with a 95 % confidence limit).

6.2.4 Tuber starch content and proportion of amylose in Prairie and Prairie *glgC-16* plants with lowered D-enzyme activity

Biochemical determination of tuber starch and amylose contents were made on freshly harvested mature tubers (table 3). The proportion of amylose was also determined in leaf starch from 9 week-old plants (table 3). There were no significant differences in the starch contents of tubers with lowered D-enzyme activity and GUS-controls, both within each plant line and between Prairie and Prairie *glgC-16* tubers (when analysed by one and two-way ANOVA with a 95 % confidence limit). A discrepancy is observed between the starch content of tubers determined enzymatically, and that determined from specific gravity values (section 6.2.3). A reason to explain this could be that starch was not completely recovered by the perchloric acid extraction, which was employed prior to the enzymatic assay. There were no significant differences in the proportion of amylose in tuber or leaf starch from plants with lowered D-enzyme activity and GUS-controls, both within each plant line and between Prairie and Prairie *glgC-16* plants (when analysed by one and two-way ANOVA with a 95 % confidence limit).

6.2.5 Sprouting characteristics of Prairie and Prairie *glgC-16* tubers with lowered D-enzyme activity

The effect of lack of D-enzyme on the onset and development of tuber sprout growth was investigated. Tubers from Prairie lines were planted 3 weeks before Prairie *glgC-16* lines. Tubers were harvested on the same day from mature plants of both lines after the foliage had become necrotic on the later-planted Prairie *glgC-16* lines. Tubers were stored for a prolonged period at 12 °C. The emergence and growth of sprouts was measured at regular intervals. Due to the different planting dates the Prairie and Prairie *glgC-16* tubers were analysed separately.

Line	Fresh weight yield (g)	Specific gravity	Dry Matter (%)	Starch (%)
PR-GUS	217.1 \pm 27.5	1.096 \pm 0.008	23.7 \pm 1.75	17.1 \pm 1.59
PR-10026-1	228.4 \pm 32.5	1.100 \pm 0.009	25.5 \pm 2.03	18.7 \pm 1.85
PR-10026-41	164.8 \pm 38.6	1.095 \pm 0.005	23.3 \pm 1.05	16.7 \pm 0.96
PR-10026-44	252.8 \pm 35.0	1.092 \pm 0.006	22.7 \pm 1.24	16.1 \pm 1.13
PT-GUS	251.3 \pm 18.5	1.093 \pm 0.006	23.0 \pm 1.21	16.4 \pm 1.47
PT-10026-59	173.8 \pm 25.9	1.092 \pm 0.007	22.8 \pm 1.61	16.2 \pm 1.47
PT-10026-101	177.5 \pm 20.6	1.090 \pm 0.067	22.4 \pm 1.42	15.8 \pm 1.29

Table 2. The tuber yields of Prairie and Prairie *glgC-16* plants with lowered D-enzyme activity and GUS control plants.

In vitro grown plantlets from Prairie and Prairie *glgC-16* lines were weaned onto soil and grown in a heated greenhouse at 20 °C, 16 h day length and at least 300 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ irradiance. Tubers were harvested from mature plants after the foliage became necrotic. Specific gravity, dry matter and starch contents were measured according to the methods described by von Scheele *et al.* (1937) in chapter 2. PR and PT indicate Prairie and Prairie *glgC-16* lines respectively. Each value is the mean of three individual plants \pm s.e.m..

Line	Tuber		Leaf*
	Starch content (mg g ⁻¹ FW)	Amylose content (%)	Amylose content (%)
PR-GUS	87.4 ± 15.4	18.4 ± 0.6	10.9 ± 1.0
PR-10026-1	78.1 ± 14.0	19.2 ± 0.9	11.5 ± 0.6
PR-10026-41	87.9 ± 25.7	18.9 ± 0.4	10.6 ± 0.6
PR-10026-44	63.8 ± 7.2	19.1 ± 0.6	9.8 ± 0.5
PT-GUS	70.3 ± 13.1	20.0 ± 0.8	10.8 ± 1.0
PT-10026-59	65.2 ± 15.0	19.8 ± 0.7	9.6 ± 0.7
PT-10026-101	71.3 ± 9.3	18.7 ± 0.9	11.5 ± 0.7

Table 3. The starch and amylose contents of leaves and tubers from Prairie and Prairie *glgC-16* plants with lowered D-enzyme activity and GUS control plants.

In vitro grown plantlets were weaned onto soil and grown to maturity in a heated greenhouse at 20 °C, 16 h day length and at least 300 µmol m⁻² sec⁻¹ irradiance. Tubers were harvested from mature plants after the foliage became necrotic. Leaves were harvested from 9 week-old plants at the end of the day. Starch and amylose content were determined biochemically as described in chapter 2. PR and PT indicate Prairie and Prairie *glgC-16* lines respectively. Values for starch content are a mean of 6 tubers, for amylose content a mean of 3 tubers and for leaf amylose each value represents the mean of 3 individual plants (five leaflets were pooled from each plant for each sample) ± s.e.m.. * Leaf starch content was not analysed.

Within the Prairie lines, between 11 and 13 weeks in storage, tubers with lowered D-enzyme activity were slightly slower at sprouting than GUS-control lines (figures 2a and b). However, after 13 weeks in storage the proportion of tubers with lowered D-enzyme activity which were sprouting was similar to GUS-control lines.

Within the Prairie *glgC-16* lines there were substantial differences in the sprouting behaviour of tubers with lowered D-enzyme activity and controls (figures 3a and b). Tubers from line 59 sprouted more quickly and the sprouts from these tubers grew more rapidly than those from GUS-control tubers. Tubers from line 101 were delayed in the onset of sprout emergence relative to line 59 and GUS-control tubers.

6.2.6 The effect of lowered D-enzyme activity on the malto-oligosaccharide content of leaves from Prairie plants during light and darkness

Nine-week old Prairie line 1 and GUS-control plants were maintained in a growth room in continuous darkness. Leaf tissue was sampled at regular intervals over a 30 h period. The plants were maintained in darkness for a further two days before being transferred to continuous light. Leaf tissue was sampled again at regular intervals over a 30 h period. Malto-oligosaccharide (MOS) extracts were prepared from each sample. MOS extracts were analysed using HPAEC by Dr Steve Coates at Advanced Technologies (Cambridge) Ltd. HPAEC traces of the MOS contents of some of the leaf tissue samples are shown in figures 4 and 5. There did not appear to be any appreciable differences in the MOS content or composition between Prairie line 1 and GUS-control plants during either darkness or light at each of the time points tested. The comparison of the MOS from leaf tissue to MOS standards did not enable all of the peaks present in the leaf samples to be identified. In some cases it was not possible to identify specific types of MOS within each sample despite reference to the known standards. To optimise this protocol for future use on potato tissue, the application of samples to ion exchange columns may enable the resolution of the MOS to be improved. Deionisation of samples has been reported to give good resolution of MOS in *A. thaliana* (Critchley, *et al.*, 2000). At the start of the dark period the D-enzyme activity in leaves from Prairie line 1 and GUS-control plants was $84.4 \pm 18.1 \text{ nmol min}^{-1} \text{ g}^{-1}$ Fresh Weight and $587.7 \pm 31.9 \text{ nmol min}^{-1} \text{ g}^{-1}$ Fresh Weight, respectively. Each value is the mean of three plants (five leaflets were pooled from each plant for each sample).

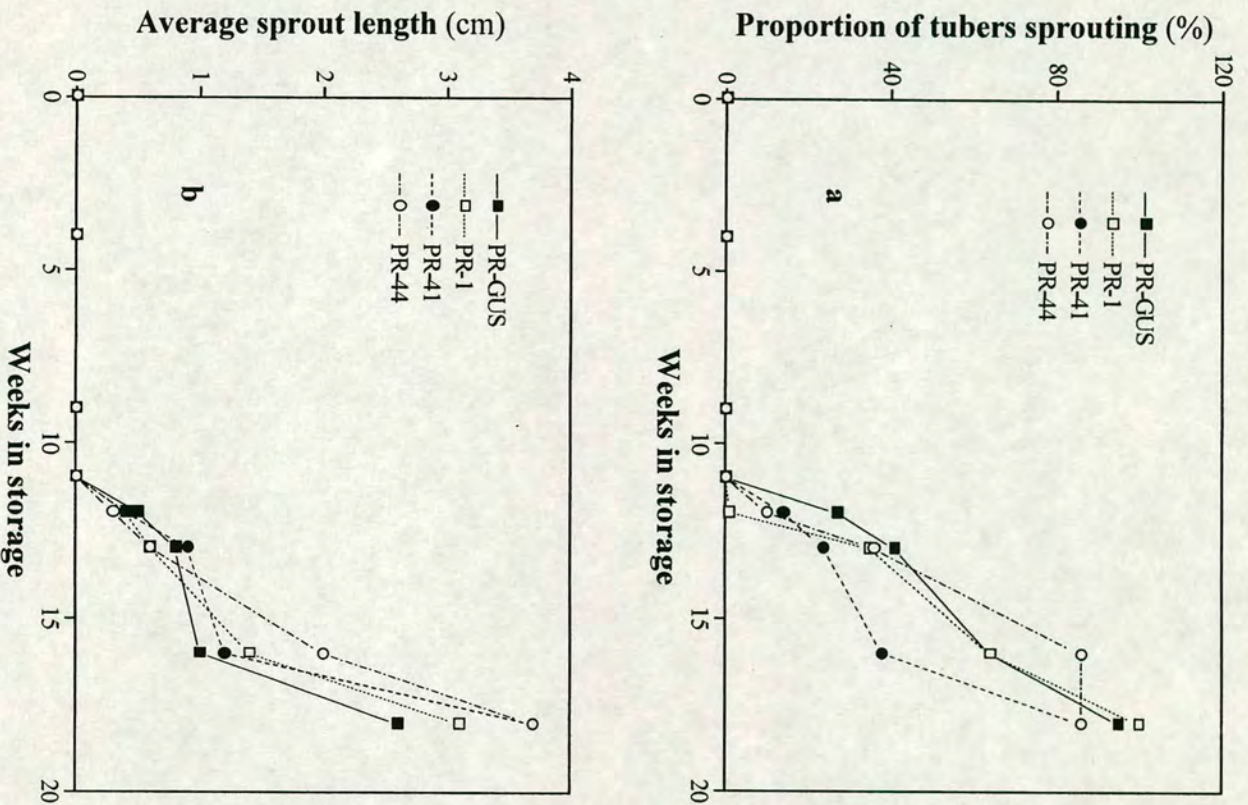


Figure 2. The development of sprouts from Prairie tubers with lowered D-enzyme activity and GUS control plants.

In vitro grown plantlets were weaned onto soil and grown in a heated greenhouse at 20 °C, 16 h day length and at least 300 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ irradiance. Tubers were harvested from mature plants after the foliage became necrotic and were placed into storage in the dark at 12 °C. The onset and development of tuber sprouting was followed over a period of 13 weeks. Each value is the mean of between 17-22 individual tubers harvested from at least 4 separate plants.

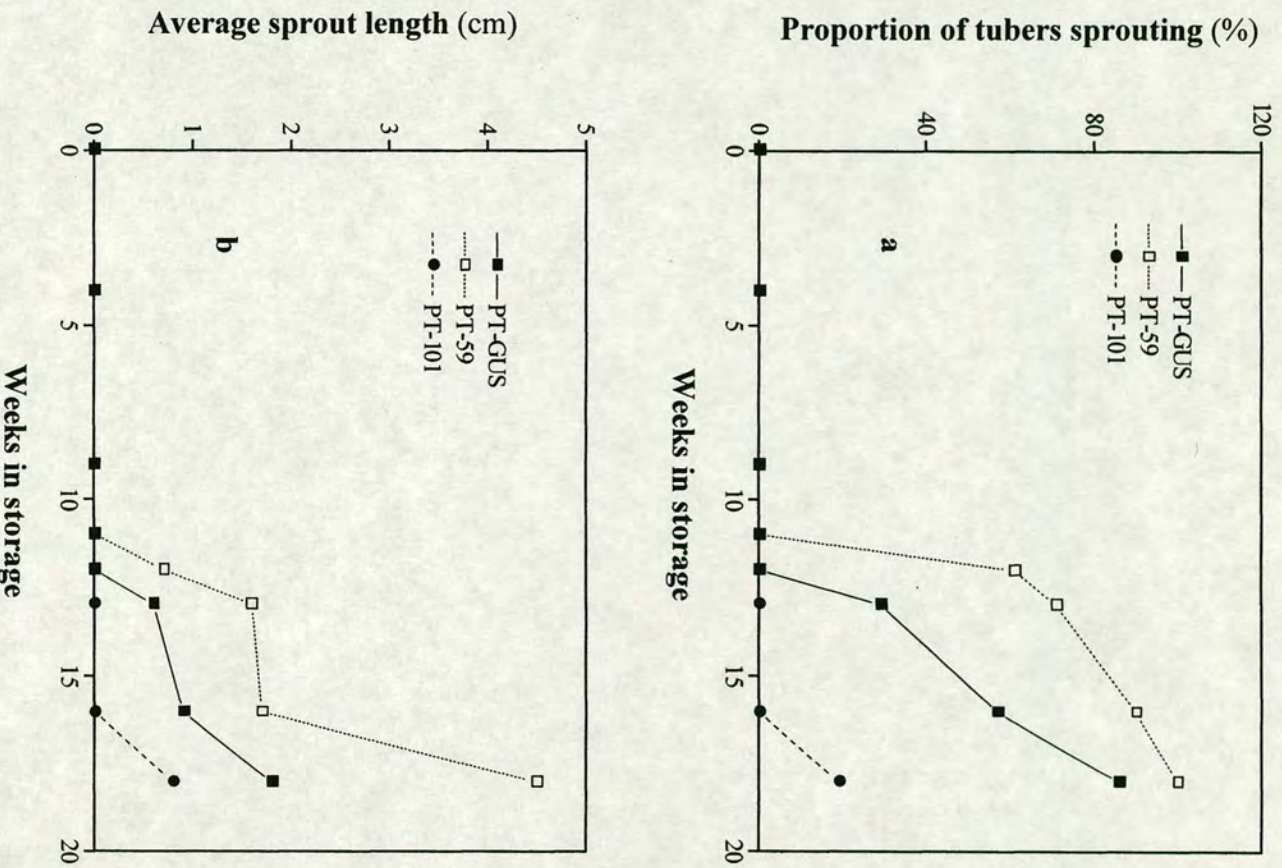


Figure 3. The development of sprouts from Prairie *glgC-16* tubers with lowered D-enzyme activity and GUS control plants.

In vitro grown plantlets were weaned onto soil and grown in a heated greenhouse at 20 °C, 16 h day length and at least 300 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ irradiance. Tubers were harvested from mature plants after the foliage became necrotic and were placed into storage in the dark at 12 °C. The onset and development of tuber sprouting was followed over a period of 13 weeks. PT indicates Prairie *glgC-16* plants. Each value is the mean of between 17-21 individual tubers harvested from at least 4 separate plants.

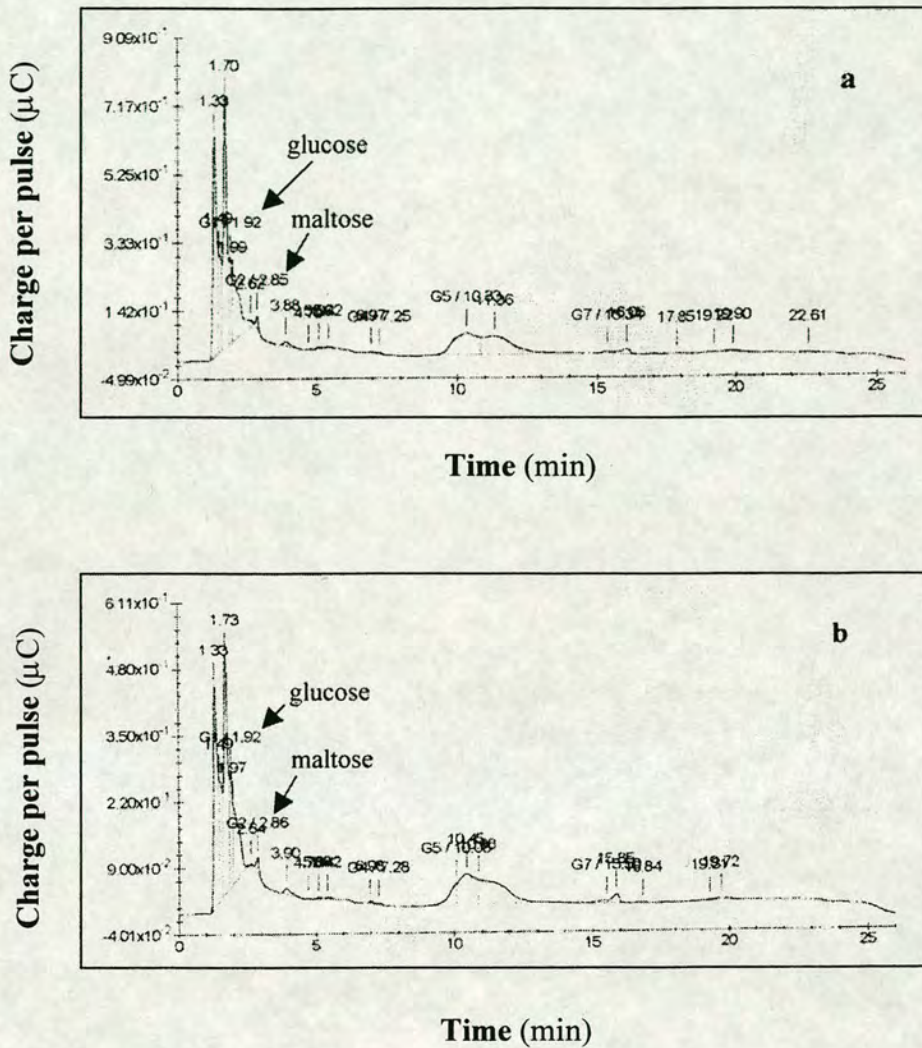


Figure 4. The malto-oligosaccharide contents of a) Prairie line 1 and b) Prairie GUS-control plants after 14 h in darkness.

Nine week-old plants were grown in random positions in a growth room under 18 h day length, $120\text{--}180 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance and 18°C . Plants were maintained in the growth room for four days before the start of the experiment to acclimatise. The plants were then grown in continual darkness at 18°C . At regular intervals leaf tissue was sampled and malto-oligosaccharides were extracted as described in chapter 2. Malto-oligosaccharide extracts were analysed at Advanced Technologies (Cambridge) Ltd. by HPAEC analysis. Glucose (1.92 min) and maltose (2.85–2.86 min) have been identified by reference to a trace of known amounts of pure saccharides (data not shown). Each trace represents the malto-oligosaccharide profile of 5 leaflets pooled from a single plant. Note: the two traces have different y-axis scales. Prairie line 1 and GUS-control leaf tissue was from 0.83 g and 0.63 g Fresh Weight respectively.

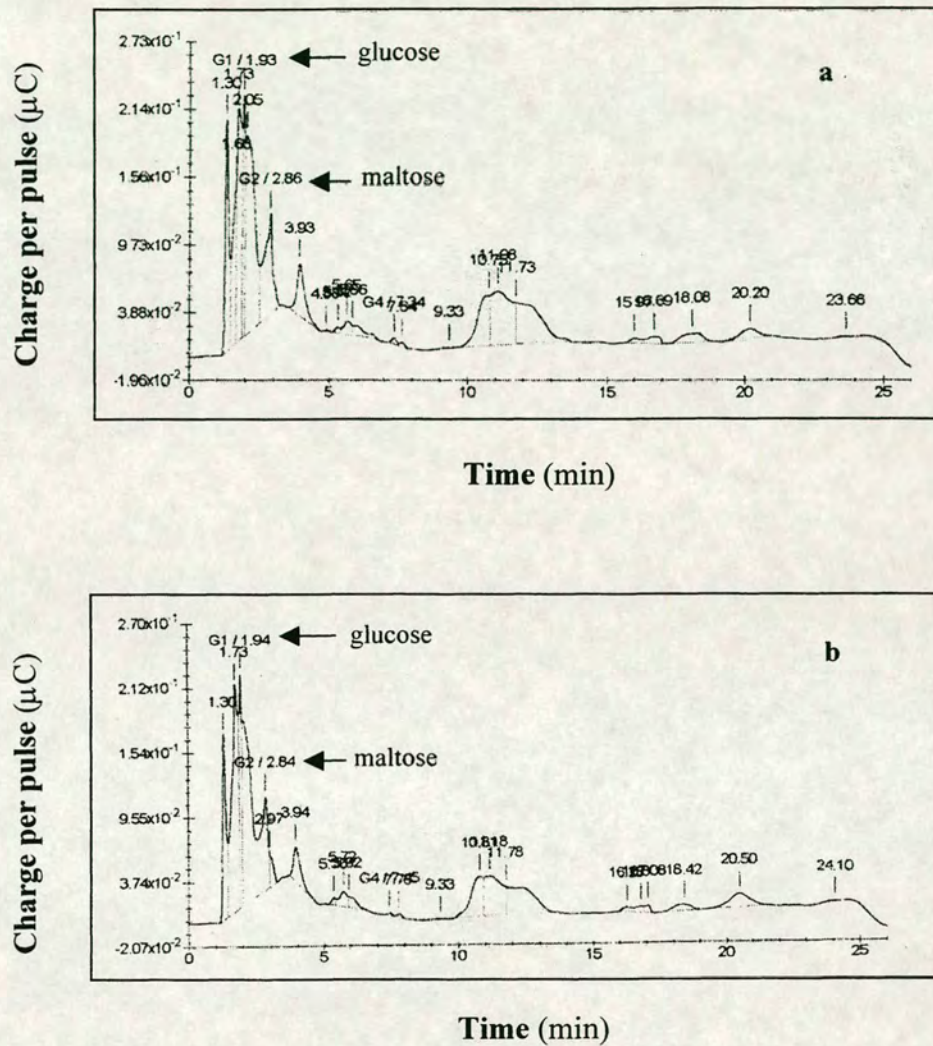


Figure 5. The malto-oligosaccharide contents of a) Prairie line 1 and b) Prairie GUS-control plants after 8 h in light.

Nine week old plants from wild type and the transgenic line P1 were maintained in darkness for a further two days after the end of the experiment described in section 6.2.7. The plants were then grown in continuous light at $120\text{--}180 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance and 18°C . At regular intervals leaf tissue was sampled and malto-oligosaccharides were extracted as described in chapter 2. Malto-oligosaccharide extracts were analysed at Advanced Technologies (Cambridge) Ltd. by HPAEC analysis. Glucose (1.93–1.94 min) and maltose (2.84–2.86 min) have been identified by reference to a trace of known amounts of pure saccharides (data not shown). Each trace represents the malto-oligosaccharide profile of 5 leaflets pooled from a single plant. Note: the two traces have different y-axis scales. Prairie line 1 and GUS-control leaf tissue was from 0.65 g and 0.80 g Fresh Weight respectively.

6.2.7 The effect of lowered D-enzyme activity on leaf starch degradation in Prairie plants during darkness

Leaf tissue was sampled from the same potato plants used for MOS analysis in section 6.2.6 and at the same time points during exposure to prolonged darkness. Each sample was assayed for starch content (figure 6). At the onset of the dark period the mean starch content of leaf tissue from line 1 was slightly greater than that from GUS-control plants (35.5 ± 1.6 mg g⁻¹ Fresh Weight compared to 32.5 ± 1.6 mg g⁻¹ Fresh Weight). Differences in the rates of starch breakdown of Prairie GUS-control and line 1 plants were small between each of the time points. Between 0 and 4 h in darkness, the average rate of starch breakdown in leaves from line 1 was approximately 5 % slower than that of control leaves. Between 0 and 8 h in darkness, the average rate of starch breakdown in leaves from line 1 was approximately 1.2 % slower than that of control leaves. After 4 h in darkness leaves from line 1 degraded starch at a similar or faster rate than control plants. At each time point leaf tissue from line 1 contained more starch than that of wild type.

6.2.8 The effect of lowered D-enzyme activity on leaf starch synthesis in Prairie plants during continuous light

Leaf tissue was sampled from the same potato plants used for MOS analysis in section 6.2.6 and at the same time points during exposure to prolonged light. Each sample was assayed for starch content (figure 7). Differences in the rates of leaf starch accumulation of Prairie GUS-control and line 1 plants were small between each of the time points. Between 0 and 12 h, the rate of accumulation of starch in line 1 plants was slightly greater than in GUS-control plants. Between 12 to 30 h in light, plants from line 1 synthesised starch at a slightly slower rate than GUS-control plants.

6.2.9 The incorporation of [U¹⁴C]sucrose into Prairie and Prairie *glgC-16* tubers with lowered D-enzyme activity

The effect of lowered D-enzyme activity on the incorporation of [U¹⁴C]sucrose into starch was investigated between Prairie line 1 and Prairie GUS-control tubers, and Prairie *glgC-16* line 59 and Prairie *glgC-16* GUS-control tubers. [U¹⁴C]sucrose was delivered via a cylindrical bore hole into developing tubers which were still attached to a mother plant. The tubers were left for a 3 h pulse period before unincorporated label was washed out and

replaced with unlabelled sucrose. Samples were removed parallel to the original bore hole at a constant distance of 5 mm. The incorporation of radiolabel into starch was determined for each sample (figure 8). There were no appreciable differences in the pattern or amount of incorporation of [^{14}C]sucrose into starch between Prairie GUS-control and line 1 tubers, and between Prairie *glgC-16* GUS-control and line 59 tubers. However, there were large differences in the incorporation of radiolabel between tubers from Prairie and Prairie *glgC-16* lines. Prairie *glgC-16* tubers continued to incorporate radiolabel up to 50 h into the chase. In Prairie tubers the radiolabel in starch increased up to around 75 h into the chase. Prairie *glgC-16* tubers incorporated approximately four times more radiolabel into starch than the Prairie tubers. After 50 h the radiolabel in starch fell at a significantly faster rate in the Prairie *glgC-16* tubers compared to the Prairie tubers. The pattern and magnitude of incorporation of radiolabel into Prairie and Prairie *glgC-16* developing tubers was similar to that described by Sweetlove *et al.* (1996b).

The experiment was repeated on mature tubers which had been stored for 8 weeks at 4 °C. The incorporation of radiolabel into starch was determined for each sample (figure 9). There were no obvious differences in the pattern and amount of [^{14}C]sucrose incorporated into starch between Prairie GUS-control and line 1 tubers, and between Prairie *glgC-16* line 59 and GUS-control tubers. There were large differences in the incorporation of radiolabel between the Prairie lines and the Prairie *glgC-16* lines. Up to approximately 120 h into the chase Prairie *glgC-16* tubers incorporated more than three times the amount of radiolabel into starch than Prairie tubers. After 100 h the radiolabel in starch fell significantly faster in the Prairie *glgC-16* tubers than in the Prairie tubers and reached a level which was similar for both plant lines after 250 h into the chase. The pattern and magnitude of incorporation of radiolabel into Prairie and Prairie *glgC-16* mature tubers was similar to that described by Sweetlove *et al.* (1996b).

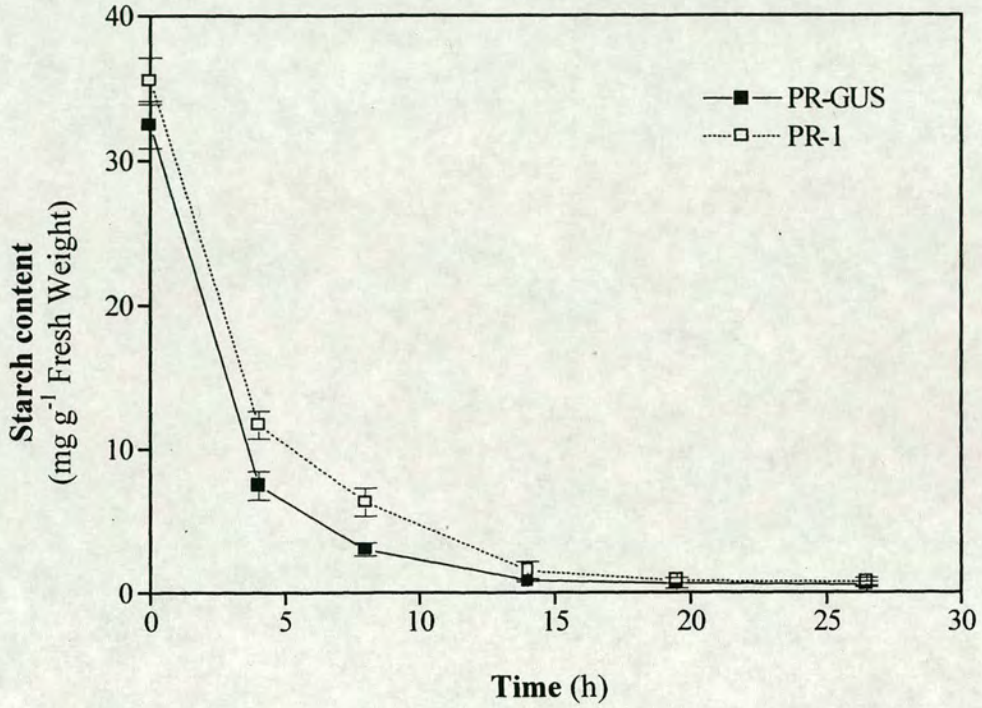


Figure 6. The starch contents of leaves from Prairie line 1 and GUS-control plants during exposure to continuous darkness.

9 week old plants were grown in random positions in a growth room under 18 h day length, 120-180 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance and 18 °C. Plants were maintained in the growth room for four days before the start of the experiment to acclimatise. The plants were then grown in continual darkness at 18 °C. At regular intervals leaf tissue was sampled and assayed for starch content as described in chapter 2. Each value is the mean of three plants (five leaflets were pooled from each plant for each sample). Error bars indicate s.e.m..

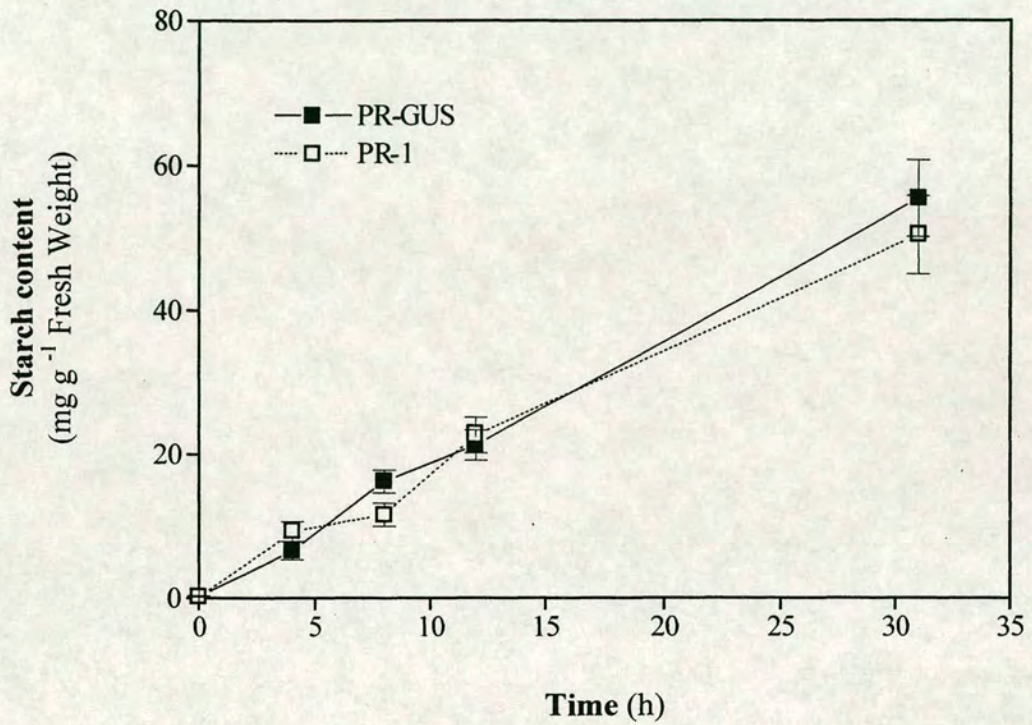


Figure 7. The starch content of leaves from Prairie line 1 and GUS-control plants during exposure to continuous light.

9 week old plants were maintained in darkness for a further two days after the end of the experiment described in section 6.2.7. The plants were then grown in continuous light at $120\text{--}180 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance and 18°C . At regular intervals leaf tissue was sampled and assayed for starch content as described in chapter 2. Each value is the mean of three plants (five leaflets were pooled from each plant for each sample). Error bars indicate s.e.m..

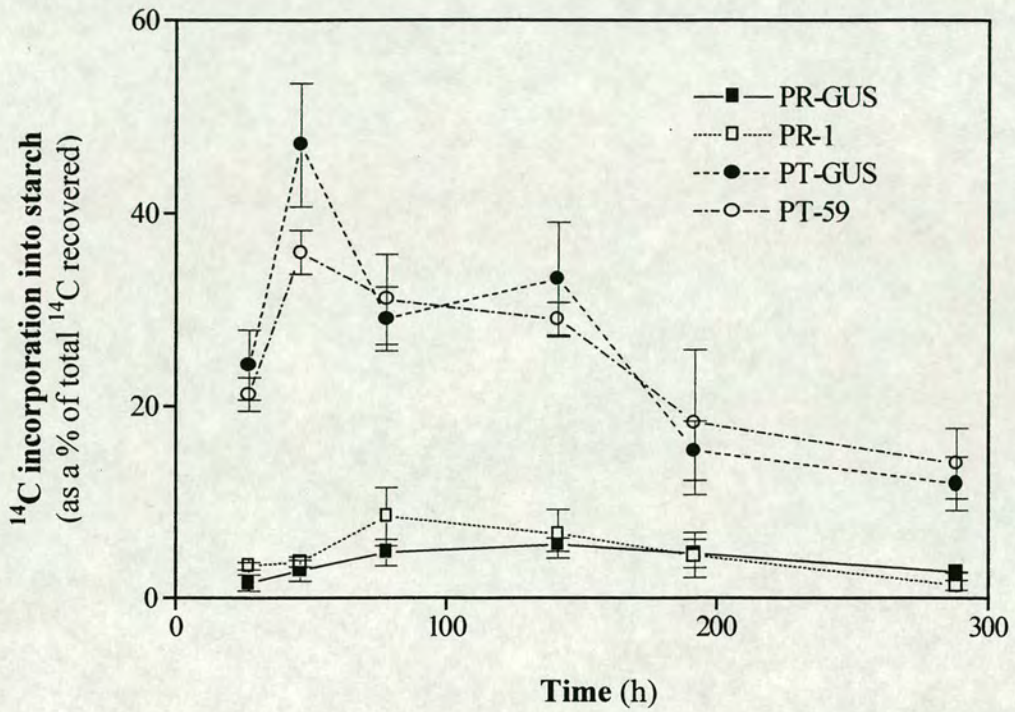


Figure 8. The incorporation of $[U^{14}C]$ sucrose into starch after a 3h pulse in Prairie line 1, Prairie *glgC-16* line 59 and GUS-control developing tubers.

$[U^{14}C]$ sucrose was delivered to developing tubers through a borehole midway between the apical and basal ends of a tuber. Care was taken to ensure that the stolon attaching each tuber to a mother plant remained intact. Plants were grown in a heated greenhouse at 20 °C, 16 h day length and at least 300 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ irradiance. A pulse of radiolabel was incubated in the tuber for 3 h, then washed out and replaced with unlabelled sucrose for the chase. Tissue was sampled and the incorporation of label into starch was measured as described in chapter 2. PR and PT indicate Prairie and Prairie *glgC-16* lines respectively. This was expressed as a percentage of the total label recovered. Each value is the mean of three individual tubers. Error bars indicate s.e.m..

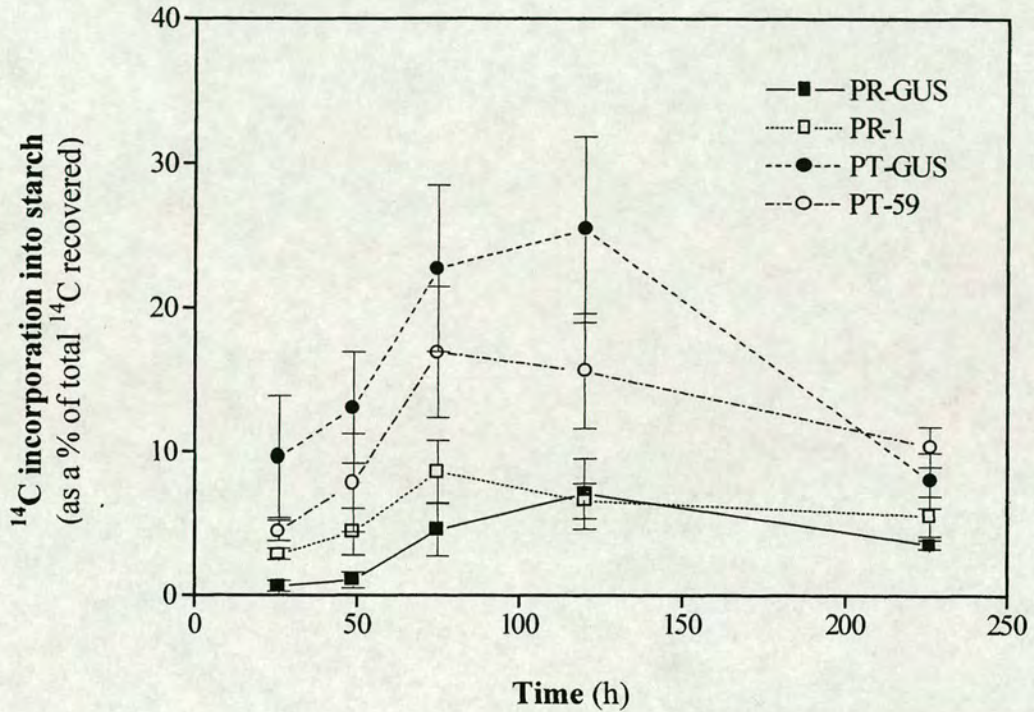


Figure 9. The incorporation of $[U^{14}C]$ sucrose into starch after a 3h pulse in Prairie line 1, Prairie *glgC-16* line 59 and GUS-control mature tubers.

Tubers were stored for 8 weeks at 4 °C. At this stage none of the tubers had started to sprout. Tubers were kept in sealed magentas in darkness at 17 °C. $[U^{14}C]$ sucrose was delivered through a bore hole midway between the apical and basal ends of a tuber. A pulse of radiolabel was incubated in the tuber for 3 h, then washed out and replaced with unlabelled sucrose for the chase. Tissue was sampled and the incorporation of label into starch was determined as described in chapter 2. PR and PT indicate Prairie and Prairie *glgC-16* lines respectively. This was expressed as a percentage of the total label recovered. Each value is the mean of three individual tubers. Error bars indicate s.e.m..

6.3 Discussion

The maximum catalytic activities of a range of starch metabolic enzymes were assayed in Prairie and Prairie *glgC-16* developing tubers. There were no significant differences in the enzyme activities within either the Prairie lines or the Prairie *glgC-16* lines, except for D-enzyme. The activity of AGPase was approximately 450 % greater in each of the Prairie *glgC-16* lines compared to the Prairie lines. The activities of β -amylase and soluble starch synthase also tended to be higher in these lines. The enzyme activities described in this study for Prairie *glgC-16* and Prairie lines are in close agreement with published data from the same lines (Sweetlove *et al.*, 1996a, b). The level of AGPase activity in the *glgC-16* expressing lines suggests that the level of expression of the *glgC-16* gene was similar to that when the same plants were analysed by Sweetlove *et al.* (1996a, b).

The effect of lack of D-enzyme on plant growth, tuber fresh weight yield, proportion of tuber dry matter and starch and tuber sprouting in Prairie and Prairie *glgC-16* lines was investigated. Plant growth, proportion of tuber dry matter, tuber starch content and proportion of amylose in leaf and tuber starch were not altered between tubers with lowered D-enzyme activity and GUS-controls within both the Prairie lines and the Prairie *glgC-16* lines. In addition, there were no differences in these same parameters between the tubers from the Prairie lines and the tubers from the Prairie *glgC-16* lines. The unaltered tuber dry matter, starch content and proportion of amylose in plants with lowered D-enzyme activity is in agreement with the effect of lack of D-enzyme in May Queen plants (chapter 3). Fresh weight yields were found to be reduced in Prairie line 41 and Prairie *glgC-16* lines 59 and 101 compared to their respective GUS-control plants, but not to a significant extent. However, in May Queen plants lacking D-enzyme, plant growth was reduced and the reduction in tuber fresh weight yield was greater than in the Prairie and Prairie *glgC-16* lines which lack D-enzyme (chapter 3). The level of D-enzyme activity in the Prairie and Prairie *glgC-16* lines was greater than in the May Queen plants; for instance Prairie line 1 and Prairie *glgC-16* line 59 contained 10.5 % and 14.4 % of wild type D-enzyme activity respectively whereas the May Queen lines E209 and E211 contained less than 1 % of wild type D-enzyme activity. It is possible that the level of D-enzyme activity in the Prairie and Prairie *glgC-16* lines was sufficient for normal plant growth. Lack of D-enzyme activity may also have had different effects in the two cultivars. This is discussed further in chapter 7.

The sprouting behaviour of tubers from Prairie lines with lowered D-enzyme activity was not appreciably different to that of Prairie GUS-control tubers. This contrasts with the significant delay in sprout emergence and sprout development in tubers from May Queen which lack D-enzyme (Takaha, 1996). The delay in sprouting of tubers from Prairie *glgC-16* line 101 is in agreement with the phenotypes of May Queen tubers. This is surprising as line 101 exhibited only a 50 % reduction in D-enzyme activity. The sprouting behaviour of Prairie *glgC-16* line 59 tubers is not readily explained. The development of sprouts on line 59 before those on the Prairie *glgC-16* GUS-control tubers does not correlate with that of the May Queen tubers with lowered D-enzyme activity. This anomalous result could be explained by somoclonal variation or by an unidentified pleiotropic effect on another enzyme(s). The sprouting behaviour of these tubers was recorded over the course of one experiment. Further investigation of the storage of tubers from line 59 tubers is therefore required to verify this observation.

Next the effect of lack of D-enzyme on leaf starch metabolism was investigated. Prairie *glgC-16* lines were not used for this analysis for two reasons. Firstly, the *glgC-16* gene is driven by a patatin promoter and therefore the starch metabolic enzymes present in the leaves of these plants may be identical to those of the Prairie lines. Secondly and conversely, it is possible that the significant alteration to tuber metabolism in these plants could have secondary effects on leaf metabolism. If so, this could make the analysis of leaf metabolism in plants which also lack D-enzyme extremely complex. The malto-oligosaccharide (MOS) content of leaves from Prairie line 1 was not affected by lack of D-enzyme during darkness and light. This is in contrast to the *A. thaliana* mutant, *dpe1-1*, in which D-enzyme activity has been eliminated. In *dpe1-1* in darkness, it was reported that there was a massive increase in MOS compared to wild type (Critchley *et al.*, 2000). In light the MOS level rapidly fell to wild type levels. The majority of the MOS which accumulated in darkness was found to be maltotriose (Critchley *et al.*, 2000). These results provided direct evidence that D-enzyme is involved in the metabolism of MOS during starch degradation in darkness. It is possible that the level of D-enzyme activity in the leaves of Prairie line 1 plants (14.4 % of wild type activity) was sufficient for the *in vivo* role(s) of the enzyme to be fulfilled. Alternatively, MOS may have been produced in the leaves of Prairie line 1 plants during darkness but could have been subsequently metabolised by the action of other enzymes. D-enzyme has been proposed to have a role in starch degradation by metabolising MOS generated by the

action of starch phosphorylase (Lin and Priess, 1988; Kakefuda and Duke, 1989). Starch phosphorylase can only metabolise MOS which are larger than maltotetraose (Steup and Schachtele, 1981). Therefore, if D-enzyme acts in conjunction with starch phosphorylase, lowering of D-enzyme activity may result in the accumulation of MOS during darkness. Amylases and α -glucosidases are capable of metabolising MOS and this alternative hydrolytic action would lead to the production of glucose rather than glucose-1-phosphate. However, the levels of glucose in the leaves of Prairie line 1 plants shown in the HPAEC traces of leaf MOS profiles were not appreciably different to Prairie GUS-controls during either darkness or light (section 6.2.6).

The plants used to sample leaves for MOS analysis were also used to investigate rates of leaf starch synthesis and degradation during light and darkness. It was found that at the start of the dark period leaf tissue from Prairie line 1 contained higher quantities of starch than leaf tissue from the GUS-control plants. A similar phenotype has also been observed in the leaves of the May Queen line E211 and in the *A. thaliana* D-enzyme mutant, *dpe1-1*, (chapter 4; Critchley *et al.*, 2000). However, only very small differences were detected in the net rates of starch accumulation and degradation between leaf tissue from line 1 and GUS-control plants. It is interesting to note that, although the differences in rates of starch breakdown were small, the leaf starch content of line 1 was greater than that of GUS-control lines at each time point. This is similar to the pattern of leaf starch breakdown in E211 (chapter 4). Lack of D-enzyme in line 1 did appear to reduce the rate of starch degradation in the early part of the dark period relative to wild type, but only very slightly. However, the cumulative effect of a small reduction in the rate of starch degradation over several diurnal cycles could result in the greater quantities of leaf starch which were observed in line 1 at the start of the dark period. This proposal is consistent with the effect of lowered D-enzyme activity on leaf starch breakdown in leaves of E211 and *dpe1-1* (chapter 4; Critchley *et al.*, 2000). Over 30 h the rate of starch synthesis in line 1 was slightly lower than that of GUS-control plants. However, the greater quantity of leaf starch in line 1 relative to GUS-control plants at the start of the dark period is not consistent with a role for D-enzyme in starch synthesis. No evidence was found to support a role for D-enzyme in leaf starch synthesis in E211 and *dpe1-1* (chapter 4; Critchley *et al.*, 2000).

Lastly, the effect of lowered D-enzyme activity on tuber starch metabolism was investigated in ^{14}C labelling experiments. There were no appreciable differences in the pattern or amount of incorporation of ^{14}C into starch between Prairie line 1 and Prairie GUS-control tubers, and between Prairie *glgC-16* line 59 and Prairie *glgC-16* GUS-control tubers. It is possible that small differences in metabolism were not detected in these tubers. Although substantial alterations to starch metabolism can be detected in *glgC-16* tubers (Sweetlove *et al.*, 1996b), high levels of variation between individual tubers could mask small metabolic differences. In addition, the level of D-enzyme activity in Prairie line 1 and Prairie *glgC-16* line 59 tubers, 10.5 % and 14.4 % of GUS-control tubers respectively, may be sufficient for the *in vivo* role(s) of D-enzyme to be fulfilled. It is also possible that lack of D-enzyme did not affect the starch metabolism of these tubers at the stages of development at which they were analysed. For example, mature tubers were analysed after being stored for 8 weeks at 4 °C to enable comparisons with the work carried out by Sweetlove *et al.* (1996b). If D-enzyme in tubers is only important during sprouting this may not have been detected in these tubers as they had not started to sprout. However, the storage of tubers at low-temperatures induces cold-sweetening which, like sprouting, is thought to result from starch degradation (Burton, 1989). If D-enzyme is involved in starch degradation during cold-sweetening then differences may have been expected between tubers lacking D-enzyme and GUS-control tubers. In addition, the method of sampling tubers may not have been representative of whole tuber metabolism; this is discussed in more detail in chapter 4. Therefore, the results from tuber labelling experiments remain inconclusive. The incorporation of ^{14}C into starch was markedly different between Prairie *glgC-16* tubers and Prairie tubers. The *glgC-16* tubers incorporated 3- to 4-fold more ^{14}C into starch than Prairie tubers. The rate of loss of ^{14}C from starch in the *glgC-16* tubers was faster than in the Prairie tubers. The metabolism of ^{14}C into starch in the *glgC-16* tubers is in close agreement with data presented in a separate study (Sweetlove *et al.*, 1996b). This indicated that the labelling experiments were reproducible and that AGPase activity had remained stable in these plants.

Chapter 7. General Discussion

7.1 The effect of lowered D-enzyme activity on tuber starch metabolism

In a previous study potato plants from the cultivar May Queen which exhibited less than 3 % of wild type D-enzyme activity were found to exhibit a reduction in tuber yield and a delay in tuber sprouting (Takaha *et al.*, 1998c). In this study these phenotypes were investigated further. It was found that a reduction in tuber fresh weight yield per plant was responsible for the reduction in yield, rather than changes in percentage dry matter and starch content. The unaltered starch content of these tubers was in agreement with Takaha *et al.* (1998c). One explanation for this reduction in tuber yield is that D-enzyme is required for tuber starch synthesis. If this is the case, then a lack of D-enzyme would result in a reduction in tuber yield. A role for D-enzyme in starch synthesis has been put forward by Colleoni *et al.* (1999b). However, the amount and structure of tuber starch was found to be unaltered in these plants (Takaha *et al.*, 1998c; chapter 3). If D-enzyme is involved in tuber starch synthesis then it may have been expected that tuber starch content or starch structure would have been altered in these plants. The extent of the reduction in tuber yield was found to be dependent on irradiance. This indicates that the activity of D-enzyme in leaf metabolism may influence tuber yield. This is discussed further in section 7.3. It was also found that the accumulation of reducing sugars in tubers during storage was delayed in plants which lacked D-enzyme. The accumulation of sugars in tubers during prolonged storage may be a result of senescent sweetening or sprouting. It is thought that starch breakdown is responsible for each of these processes (Burton, 1989). The delay in sugar accumulation in these tubers could have been caused by a disruption to starch degradation during senescent sweetening, sprouting or both. The accumulation of sugars during storage devalues the use of a potato crop for food industries such as crisp and chip production. A delay in the accumulation of sugars in tubers is therefore a desirable trait with high economic potential. A significant amount of scientific research has been directed towards achieving this objective (e.g. Brown *et al.*, 1990; Cottrell *et al.*, 1993).

The reduction in tuber fresh weight yield and the delay in tuber sprouting indicates that lack of D-enzyme does alter tuber metabolism in these plants. To investigate the exact role of D-

enzyme in tubers, starch metabolism was analysed in labelling experiments. No obvious differences could be detected in rates of starch synthesis or starch turnover between tubers with lowered D-enzyme activity and controls in either developing or sprouting tubers. One reason for this could have been that the labelling experiments were not sensitive enough to detect small differences. To address this, D-enzyme activity was lowered in transgenic potato plants from the cultivar *Prairie* which were reported to exhibit exaggerated rates of starch synthesis and turnover due to the expression of the *glgC-16* gene from *E. coli*. It was hypothesised that differences in starch metabolism due to lack of D-enzyme would be more likely to be resolved in tubers from these plants. Surprisingly, no differences between the starch metabolism of tubers with exaggerated rates of starch turnover and lowered D-enzyme activity and control tubers were detected. However, the level of D-enzyme activity in these tubers was 14 % of that of wild type and this could have been high enough for the *in vivo* role(s) of the enzyme to be fulfilled. It was possible that the sampling of tissue from 1 mm bore holes in the labelling experiments was not an adequate way of representing whole tuber metabolism and local differences within the tuber tissue may not have been represented in the analysis. To address this, the localisation of reducing sugars could be analysed by studying tissue prints from tubers. This would give an initial indication of the occurrence of local differences in carbohydrate metabolism in tubers. It may also be interesting to investigate the metabolism of ^{14}C -sucrose after it has been supplied to the tissue immediately surrounding tuber eyes. Tuber eyes are the sites of sprout emergence. Therefore differences in rates of starch mobilisation may be more likely to be detected around eyes than in other regions. It is possible that the observed reduction in tuber yield and delay in sprouting of *May Queen* tubers may have been a result of only very small reductions in rates of starch synthesis or turnover. If this was the case then it is likely that the variation between individual tubers was high enough to prevent these differences from being detected, whatever the experimental system employed. One way of addressing this would be to analyse tubers which are as uniform as possible. *In vitro* maintained tubers may be more uniform than soil-grown tubers and could therefore be a more suitable system. In addition, feeding $^{14}\text{CO}_2$ to whole plants and then analysing rates of tuber starch synthesis and breakdown would be a way of supplying label more uniformly to tubers. Alternatively, it may be necessary to lower D-enzyme activity to levels which are less than have been achieved in the *May Queen*, *Prairie* and *Prairie glgC-16* plants described in this study, before differences in tuber metabolism can be observed.

It is also important to consider that if lack of D-enzyme activity did affect rates of tuber starch degradation or synthesis then this could have been compensated for by the alteration of other enzymatic activities. For example, if lack of D-enzyme had resulted in a reduction in the levels of starch turnover in *glgC-16* tubers this could have resulted in a high starch phenotype of these tubers. However, any reports describe attempts to increase tuber yield by manipulation of sink tissue metabolism which have not been successful due to the flexible nature of plant metabolism. Plants have been reported to respond to perturbations in metabolism by using alternative or redundant enzymes, adjusting existing enzyme activities or altering intracellular and extracellular partitioning (Sonnewald and Herbers, 1999). The conclusion of many groups has been that plant metabolism needs to be better understood before attempts are made to manipulate specific traits. The activities of other starch metabolic enzymes were not found to be affected by lack of D-enzyme in Prairie *glgC-16* tubers compared to GUS-control tubers. However, a comprehensive investigation of the occurrence of compensatory effects was not undertaken in this study.

7.2 The effect of lowered D-enzyme activity on leaf starch metabolism

The function of D-enzyme in leaf starch metabolism was investigated in the cultivars May Queen and Prairie. The starch contents of leaves during light and darkness were measured over a defined time course. For both cultivars, lack of D-enzyme did not appear to affect the rate of starch synthesis during light. This is consistent with the phenotype of a D-enzyme mutant of *A. thaliana*, *dpe1-1*, (Critchley *et al.*, 2000). In *dpe1-1*, amylopectin structure is unaltered in the absence of D-enzyme. An analysis of the structure of amylopectin from potato leaf starch was not undertaken in this study. Therefore, no evidence was found to either support or refute a role for D-enzyme in starch synthesis as proposed by Colleoni *et al.* (1999b). An investigation of the structure of leaf starch from potato plants with lowered D-enzyme activity is an important experiment to be undertaken in the future. In leaf tissue from the May Queen line E211 starch degradation was slightly reduced during darkness. A similar trend was observed in leaf tissue from Prairie line 1, but in this case, the reduction in rate of starch degradation was very small. These results are consistent with those from *dpe1-1* and suggest that D-enzyme in potato leaves may have a role in starch degradation. In *dpe1-1*, it

was also found that malto-oligosaccharides (MOS) accumulated massively during darkness, compared to controls. This is strong evidence to suggest that D-enzyme is involved in the metabolism of MOS in *A. thaliana* during starch degradation in darkness. The MOS content of Prairie leaves with lowered D-enzyme activity was not appreciably altered from controls. It is possible that if MOS did accumulate in the leaves of these plants then it could have been metabolised by other enzymes and therefore would not have been detected. Alternatively, the levels of D-enzyme activity in the leaves of the Prairie plants may have been high enough for the enzyme to fulfil its *in vivo* role(s). The analysis of the MOS content of May Queen leaves was initiated but not completed at the time of writing. The way in which lack of D-enzyme in potato leaves resulted in an apparent reduction in starch degradation was not investigated. Based on the results from *dpe1-1*, it has been suggested that either the accumulation of MOS during darkness was responsible for inhibiting starch degradation or that D-enzyme was directly involved in degrading starch molecules, or both.

Many reports describe disruptions to leaf metabolism which are partially compensated for by other enzymatic activities (see chapter 4 discussion). In addition, there is evidence that plants contain surplus photosynthetic potential which may be used on demand (Dwelle, 1990). In this study the occurrence of potential compensatory effects in response to lack of D-enzyme in leaf tissue was not investigated. This is something which would be important to address in the future. If compensatory effects are occurring it may be that lack of D-enzyme actually results in more severe metabolic phenotypes than were observed in this study and by Takaha *et al.*, (1998c).

7.3 The influence of D-enzyme in leaves on the metabolism of tubers

Transitory leaf starch is synthesised and degraded according to periods of illumination and this is not complicated to a significant extent by concurrent starch turnover. The diurnal fluctuations in starch synthesis and degradation make transitory starch an ideal system in which to study the effect of D-enzyme on starch synthesis and degradation. In contrast, the analysis and sampling of storage starch is more complex (section 7.1). Large amounts of starch and high levels of variation between tubers make small differences between tubers

difficult to resolve. This may even explain why in plants with lowered D-enzyme activity differences were suggested in the starch metabolism of leaves but not tubers.

The evidence presented in this work suggests that D-enzyme activity in leaves may influence tuber metabolism. A disruption of leaf metabolism could affect tuber metabolism by altering carbon flux through a plant. Recent work on sink - source relations in potato has shown that reactions in source tissue can account for up to 90 % of the control of carbon flux in a plant (Sweetlove *et al.*, 1998; Sweetlove and Hill, 2000). In May Queen plants with lowered D-enzyme activity the reduction of tuber yield was found to be due to a lowering of fresh weight yield per plant rather than to changes in percentage dry matter and starch content. This is consistent with a lack of D-enzyme resulting in a reduction in the export of carbon from leaf tissue. To investigate this further, $^{14}\text{CO}_2$ could be supplied to the leaves of intact plants and the flux of carbon out of leaves could be measured. This would show whether rates of leaf carbon export were reduced in plants lacking D-enzyme. It remains unclear why irradiance should affect the extent of the reduction in tuber fresh weight yield. Further investigation is required to show whether this may be due to changes in the expression of the transgene.

7.4 Conclusion

From the work presented in this study and that undertaken previously on D-enzyme in potato and *A. thaliana* it is evident that the severity of many of the phenotypes associated with lack of D-enzyme in these plants correlates closely with the amount of D-enzyme present in the tissue. For example, a complete lack of D-enzyme in *A. thaliana* apparently resulted in a greater reduction in plant growth than the 2 % of D-enzyme activity did in potato (Critchley *et al.*, 2000; Takaha *et al.*, 1998c; chapter 3). In addition, in May Queen tubers with lowered D-enzyme activity, line E209 has the greatest reduction in D-enzyme activity and is the slowest transgenic line to sprout; it also exhibits the largest reduction in yield and slower growth than the other transgenic lines compared to controls. This dose - effect relationship strengthens the association between lack of D-enzyme and the observed phenotypes of these plants. This also indicates that the level of D-enzyme activity in Prairie plants which was higher than that of the May Queen lines may account for failure to detect significant differences in the tuber yield and tuber sprouting of these plants. However, it is important to mention that the effect of transgenes can differ markedly between cultivars. For instance, in

contrast to expression of the *glgC-16* gene in Prairie tubers, expression of the *glg-16* gene in the cultivar Russet Burbank was found to result in an increase in tuber starch content of approximately 30 % (Stark, *et al.*, 1992).

Based on the suggestion that D-enzyme in leaf starch metabolism affects tuber metabolism it is now essential that the effect of organ specific expression of D-enzyme activity is investigated. The grafting technique used to engineer plants with organ specific expression of D-enzyme activity did not find any differences between plants which exhibited D-enzyme activity in leaves or tubers only. However, there were several experimental problems associated with this technique. Producing plants with leaf or tuber specific expression of D-enzyme achieved through molecular methods may be a more effective approach. To address this, a plasmid construct designed to be used to lower D-enzyme activity in tubers has been produced for future use. In addition, it is possible that D-enzyme acts in conjunction with other enzymes *in vivo*. Lowering D-enzyme activity and other enzymes of starch degradation in the same plants could be highly informative. This has proved successful for studying other enzymes of starch metabolism. For example, a reduction in SBE A activity in potato to 5 % of wild type activity had little effect on tuber metabolism. When the SBE A and SBE B were both reduced a dramatic affect on starch structure was observed (Safford *et al.*, 1998; Schwall *et al.*, 2000). Lastly, one of the most important questions remaining to be resolved in work on D-enzyme is the nature of the substrate for the enzyme *in vivo*.

It is clear from work on *A. thaliana* and potato that the manipulation of D-enzyme activity can have a profound effect on starch metabolism. In this study it was shown that D-enzyme may effect sink-source relations. It was also shown that lack of D-enzyme can potentially be used to modify characters of key economic importance in potato. The delayed sprouting and possible delay in senescent sweetening of tubers lacking D-enzyme could provide a cheaper alternative to the use of chemical sprout suppressants which is the present practice for controlling tuber behaviour during storage. Further investigation of D-enzyme and other starch metabolic enzymes may result in the future manipulation of the productivity of crop plants such as potato and, in addition, the engineering of starch with structures designed for specific uses.

Appendix

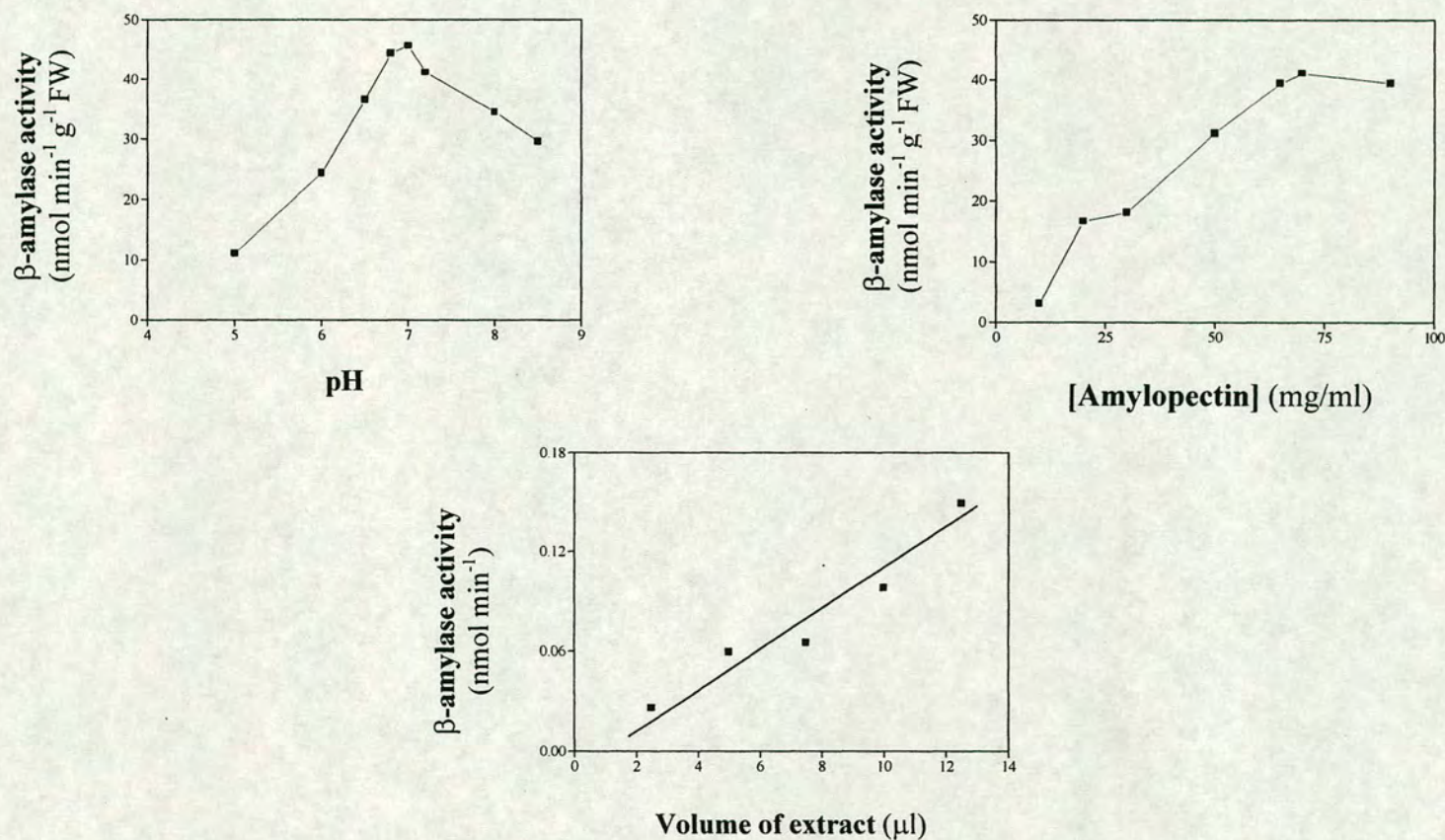


Figure 5. The optimisation of the β -amylase activity assay in potato tuber extracts.

Potato tubers were harvested at the end of a growing season and stored for 8 weeks at 4 °C. Tubers were selected and assayed for β -amylase activity as described in chapter 2. The assay was optimised with respect to pH, amylopectin concentration and amount of extract. Parameters were varied to enable maximum catalytic activity to be measured.

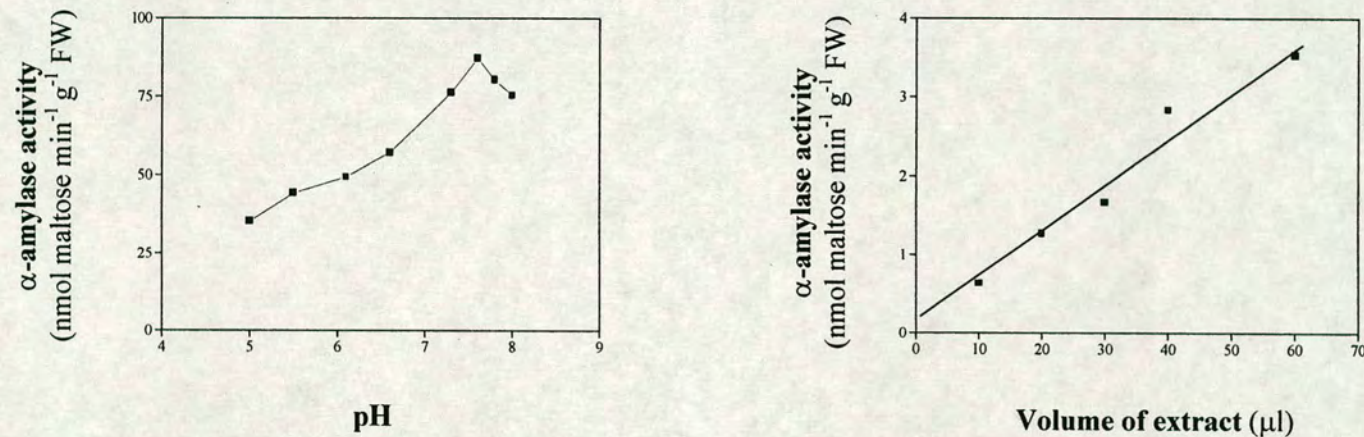


Figure 4. The optimisation of the α -amylase activity assay in potato tuber extracts.

Potato tubers were harvested at the end of a growing season and stored for 8 weeks at 4 °C. Tubers were selected and assayed for α -amylase activity as described in chapter 2. The assay was optimised with respect to pH and amount of extract. Parameters were varied to enable maximum catalytic activity to be measured.

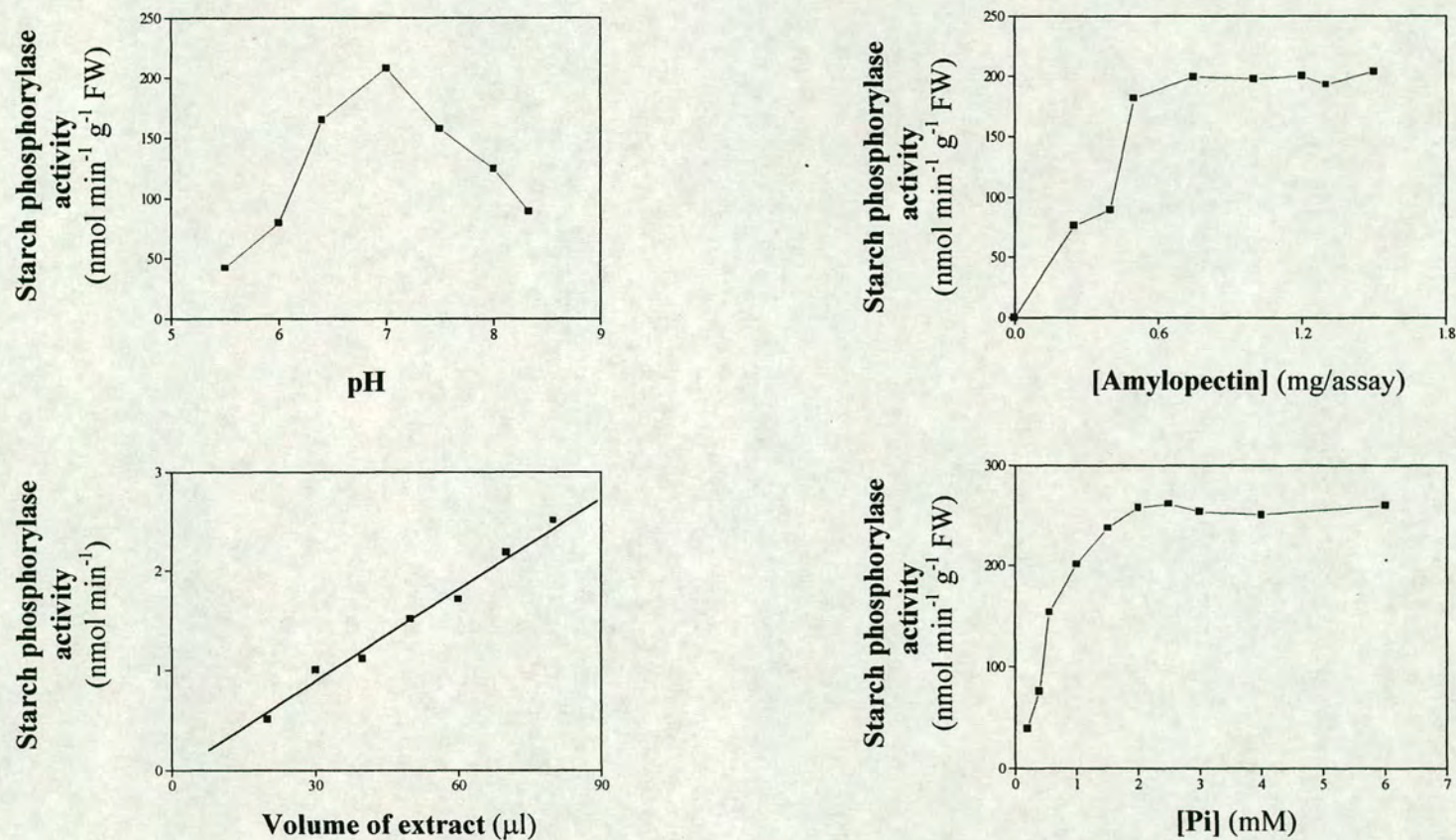


Figure 3. The optimisation of the starch phosphorylase activity assay in potato tuber extracts.

Potato tubers were harvested at the end of a growing season and stored for 8 weeks at 4 °C. Tubers were selected and assayed for starch phosphorylase activity as described in chapter 2. The assay was optimised with respect to pH, amylopectin concentration, amount of extract and amount of Pi. Parameters were varied to enable maximum catalytic activity to be measured.

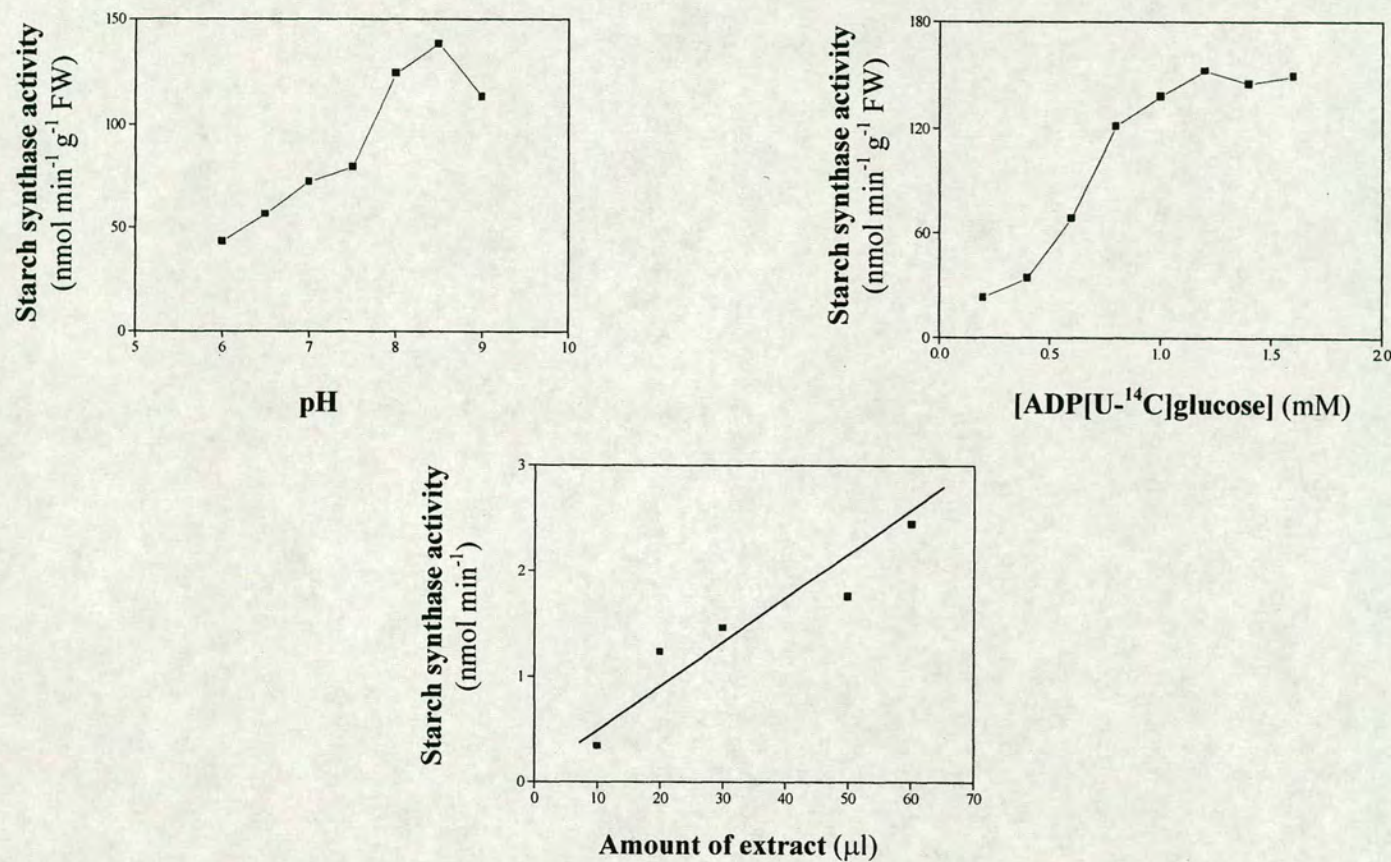


Figure 2. The optimisation of the soluble starch synthase activity assay in potato tuber extracts.

Developing potato tubers were harvested from 9-week old plants and assayed for soluble starch synthase activity as described in chapter 2. The assay was optimised with respect to pH, ADP[U-¹⁴C]glucose concentration and amount of extract. Parameters were varied to enable maximum catalytic activity to be measured.

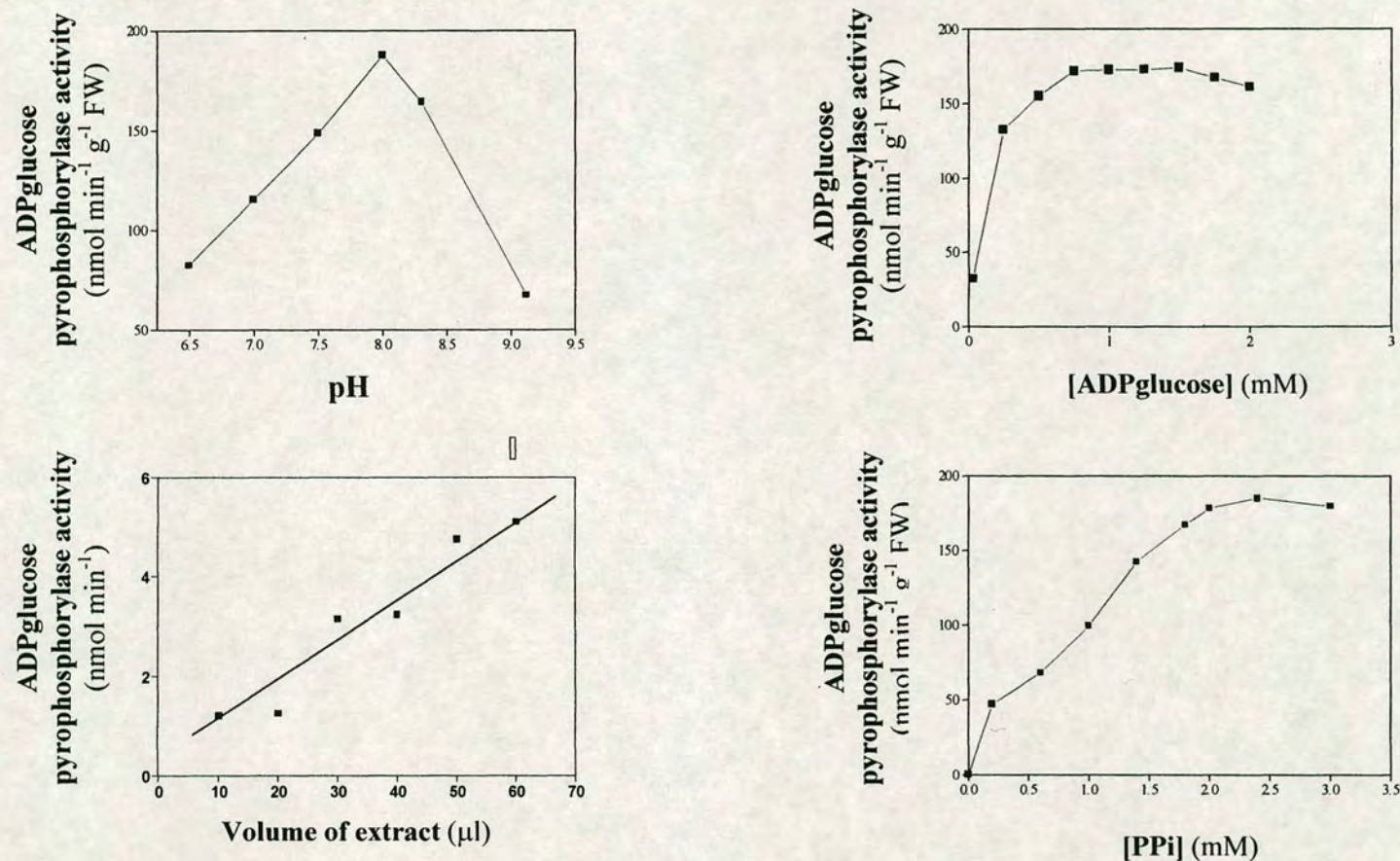


Figure 1. The optimisation of the ADPglucose pyrophosphorylase activity assay in potato tuber extracts.

Potato tubers were harvested at the end of a growing season and stored for 8 weeks at 4 °C. Tubers were selected and assayed for AGPase activity as described in chapter 2. The assay was optimised with respect to pH, ADPglucose concentration, amount of extract and amount of PPi. Parameters were varied to enable maximum catalytic activity to be measured.

Bibliography

- Abbot I. R., Matheson N K.** (1972) Starch depletion in germinating wheat, wrinkled-seed peas and senescing tobacco leaves. *Phytochemistry* **11**: 1261-1272.
- Abel G.J.W., Springer F., Willmitzer L., Kossmann J.** (1996) Cloning and functional analysis of a cDNA encoding a novel 139 KDa starch synthase from potato (*Solanum tuberosum* L.). *Plant J.* **10**: 981-991.
- Akhavan-Tafti H., Katsuaki S., Arghavni Z., DeSilva R., Handley R S., Sugioha Y., Eickhart R A., Perkins M P., Schaap A P.** (1995a) Lumigen ® RS: Chemiluminescent detection of horseradish-peroxidase by enzymatic generation of acridinum esters. *Clinical Chem.* **41**:1368-1369.
- Akhavan-Tafti H., Schaap A P., Arghvani Z., DeSilva R., Eickhart R A., Handley R S.,et al.,** (1995b) CCD camera imaging for the chemiluminescent detection of enzymes using ultrasensitive reagents. *Biolum. Chemilum. Funds. Appl. Aspects.* 199 – 202.
- Albersheim P., Darvill A.G., McNeil M et al.,** (1983) Oligosaccharins, naturally occurring carbohydrates with biological regulatory functions. In Structure and Function of Plant Genomes, Volume III (Ciferi O. and Dure L., eds). New York: Plenum, pp 293-312.
- ap Rees T., Beevers H.** (1960) Pathways of glucose dissimilation in carrot slices. *Plant Physiol.* **35**: 830-839.
- ap Rees T., Fuller W.A., Wright B. W.** (1977) Measurements of glycolytic intermediates during the onset of thermogenesis in the spadix of *Arum maculatum*. *Biochim. Biophys. Acta* **461**: 274-282.
- ap Rees T., Morrell S.** (1990) Carbohydrate metabolism in developing potatoes. *Am. Potato J.* **67**: 835-847.
- Badenhuizen N.P.** (1969) *The biogenesis of starch granules in higher plants*. Appleton-Century Crofts, New York, USA.
- Ball S.G., Dirick L., Decq A., Martiat J.C., Matagne R.F.** (1990) Physiology of storage starch in the monocellular algae *Chlamydomonas reinhardtii*. *Plant Sci.* **66**: 1-9.
- Ball S., Guan H.P., James M., Myers A., Keeling P., Mouille G., Buleon A., Colonna P., Priess J.** (1996) From glycogen to amylopectin: a model for the biogenesis of the plant starch granule. *Cell* **86**: 349 -352.

Ball S G., van de Wal M H B J., Visser R G F. (1998) Progress in understanding the biosynthesis of amylose. *Trends Plant Sci.* **3**: 462-467.

Baxter E D., Duffus C M. (1973) Phosphorylase activity in relation to starch synthesis in developing *Hordeum distichum* grain. *Phytochemistry* **12**: 2321-2330.

Beck E. (1985) The degradation of transitory starch in chloroplasts. In *Regulation of carbon partitioning in photosynthetic tissue*. (Heath R.L., Priess J. editors), Waverly Press, Baltimore USA, p. 27-44.

Beck E., Ziegler P. (1989) Biosynthesis and degradation of starch in higher plants. *Ann Rev Plant Physiol. Mol. Biol.* **40**: 95 - 117.

Bevan M. (1984) Binary *Agrobacterium* vectors for plant transformation. *Nuc. Acid Res.* **12**: 8711-8721.

Blundy K.S., Blundy M.A.C. Carter D., Wilson F., Park W.D., Burrell M.M. (1991) The expression of class I patatin gene fusions in transgenic potato varies with both gene and cultivar. *Plant Mol. Biol.* **16**: 153-160.

Bocca., J (1997) Initiation of starch biosynthesis. Purification and characterisation of UDPglucose: protein transglycosylase from potato tubers. *Plant Phys. Biochem.* **35**: 205-212.

Bradford M M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein dye-binding. *Anal. Biochem.* **72**: 248-254.

Britz S.J. (1990) Regulation of photosynthate partitioning into starch in soybean leaves. Response to natural daylight. *Plant Physiol.* **94**: 530-556.

Brown J., Mackay G R., Bain H., Griffith D W., Allison M J. (1990) The processing potential of the cultivated potato, *Solanum tuberosum* L., after storage at low temperatures. 2. Sugar accumulation. *Potato Res.* **33**: 29-227.

Burton R A., Bewley J D., Smith A M., Bhattacharyya M K., Tatge H., Ring S., Bull V., Hamilton W D O., Martin C. (1995) Starch branching enzymes belonging to distinct enzyme families are differentially expressed during pea embryo development. *Plant J.* **7**: 3-17.

Burton W G., Wilson A R. (1978) The sugar content and sprout growth of tubers of cv. Record, grown in different localities, when stored at 10°C, 20°C and 20°C. *Potato Res.* **21**: 269-83.

Burton W.G. (1989) The Potato. 3rd Edition, Longman Scientific & Technical.

Burton W.G., van Es A., Hartmans K J. (1992) The Physics and physiology of storage. In; P. M. Harris (ed.), The Potato Crop, The Scientific Basis for Crop Improvement. Chapman and Hall, pp 608-727.

Cao H., Sullivan T.D., Boyer C.D., Shannon J.C. (1995) *Bt1* a structural gene for the major 39-44 kDa amyloplast membrane polypeptides. *Physiol. Plantarum* **95**: 176 - 186.

Cattaneo J., Damotte M., Sigal N., Sanchez-Medina F., Puig J. (1969) Genetic studies of *Escherichia coli* K12 mutants with alterations in glycogenesis and properties of an altered adenosine diphosphate glucose pyrophosphorylase. *Biochem. Biophys. Res. Comms.* **34**: 694 - 701

Chang C W. (1979) Starch and its component ratio in developing cotton leaves. *Plant Physiol.* **63**: 973-977.

Chang C. W. (1980) Starch depletion and sugars in developing cotton leaves. *Plant Physiol.* **65** 844-847.

Chapman G W., Jr., Pallas J E., Jr. Mendicino J. (1972) The hydrolysis of maltodextrins by a β amylase isolated from leaves of *Vicia faba* L. *Biochem. Biophys. Acta.* **276**: 491 - 507.

Chourney P.S., Taliencio E.W. (1994) Epistatic interaction and functional compensation between the two tissue and cell-specific sucrose synthase genes in maize. *Proc. Nat. Acad. Sci. USA* **91**: 7917 - 7921.

Clarke B R., Denyer K., Jenner C F., Alison A M. (1999) The relationship between the rate of starch synthesis, the adenosine 5'-diphosphoglucose concentration and the amylose content of starch in developing pea embryos. *Planta* **209**: 324-329.

Classen P A M., Budde M A W., Van Calker M H. (1993) Increase in phosphorylase activity during cold-induced sugar accumulation in potato tubers. *Potato Res.* **36**: 205-217.

Cochrane M P., Duffus C M., Allison M J M Mackay G R. (1991a) Amylolytic activity in stored potato tubers. 1. Estimation using *p*-nitrophenyloligosaccharides. *Potato Res.* **34**: 325-341.

Cochrane M P., Duffus C M., Allison M J M Mackay G R. (1991b) Amylolytic activity in stored potato tubers. 2. The effect of low temperature storage on the activities of α -amylase and α -glucosidase in potato tubers. *Potato Res.* **34** : 333-341.

Cohn M. (1961) Phosphorylases (survey). In: The enzyme, vol. V. (Boyer P.D., Lardy H., Mybrack K., eds.) Academic Press, New York. pp. 179 - 206.

Coleman W.K., King R. R. (1984) Changes in endogenous levels of abscisic acid, soluble sugars and proline levels during tuber dormancy in *Solanum tuberosum* L. *Am. Potato J.* **61**: 437-449.

Colleoni C., Dauville D., Mouille G., Buleon A., Gallant D., Bouchet B., Morrell M., Samuel M., Delrue B., d'Hulst C etc (1999a). Genetic and biochemical evidence for the involvement of α -1,4 glucanotransferases in amylopectin synthesis. *Plant Physiol.* **120**: 993-1003.

Colleoni C., Dauville D., Mouille G., Morell M., Samuel M., Slomiany M-C., Lienard L., Wattebled F., d'Hulst C., Ball S., (1999b) Biochemical characterisation of the *Chlamydomonas reinhardtii* α -1,4 glucanotransferase supports a direct function in amylopectin biosynthesis. *Plant Physiol.* **120**: 1005-1014.

Conrads J., van Berkel J., Schachtele C., Steup M. (1986); Non-chloroplast α -D 1,4 glucan phosphorylase from pea leaves: characterisation and in-situ localisation by indirect immunofluorescence. *Biochim. Biophys. Acta* **882**: 452 - 463.

Cossins E A.. (1980) One-carbon metabolism. In: The Biochemistry of Plants – A Comprehensive Treatise, Vol II (Davis D D ed) Academic Press, New York. pp 365-418.

Cottrell J E., Duffus C M., Paterson L., Mackay G R., Allison M J., Bain H. (1993) The effect of storage temperature on reducing sugar concentration and the activities of three amylolytic enzymes in tubers of the cultivated potato, *Solanum tuberosum* L. *Potato Res.* **36**: 107-117.

Craig J., Lloyd J.R., Tomlison K., Barber L., Edwards A., Wang T.L., Martin C., Hedley C., Smith A.M. (1998) Mutations in the gene encoding starch synthase II profoundly alter amylopectin structure in pea embryos. *Plant Cell* **10**: 413 - 426.

Critchley J H. (1999) Investigation of the role of disproportionating enzyme in starch metabolism by isolation and characterisation of a mutant of *Arabidopsis thaliana* (L.) Ph.D. Thesis, University of Edinburgh.

Critchley J H., Zeeman SC., Takaha T., Smith A M., Smith S M. (2000) A critical role for disproportionating enzyme in starch breakdown but not in starch synthesis is revealed by a knock-out mutation in *Arabidopsis*. *Planta* (In press).

Dale P.J., Hampson K.K. (1995) An assessment of morphogenic and transformation efficiency in a range of varieties of potato (*Solanum tuberosum* L.) *Euphytica* **85**: 101 - 108.

Davies H V., Ross H A. (1987) Hydrolytic and phosphorolytic enzyme activity and reserve mobilisation in sprouting tubers of potato (*Solanum tuberosum* L.). *J. Plant Physiol.* **126**: 387-397.

Davies H V. (1990) Carbohydrate metabolism during sprouting. *Am. Potato J.* **67**: 815 – 827.

Davies H V., Viola R. (1988) The effect of gibberellic acid on starch breakdown in sprouting tubers of *Solanum tuberosum* L. *Annals Botany* **61**: 689-693.

Deckert., G Warren P V., Gaasterland T., Young W G., Lenox AL., Graham D E., Overbeek R., Snead M A., Keller M., Aujay M., Huber R., Feldman R A., Short J M., Olson G J., Swanson R V. (1998) The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. *Nature* **392**: 353-558.

Denyer K., Dunlap F., Thorbjornsen T., Keeling P., Smith A.M. (1996a) The major form of ADP-glucose pyrophosphorylase in maize endosperm is extra-plastidial. *Plant Physiol.* **112**: 779-785.

Denyer, K., Clarke, B, Hylton, C, Tatge, H, Smith, A M. (1996b) The elongation of amylose and amylopectin chains in isolated starch granules. *Plant J.* **10**: 1135-1143

Denyer K., Waite D., Edwards A., Martin C., Smith A M. (1999) Interaction with amylopectin influences the ability of granule-bound starch synthase I to elongate malto-oligosaccharides. *Biochem. J.* **342**: 647-653.

Dixon W.L., ap Rees T. (1980) Carbohydrate metabolism during cold-induced sweetening of potato tubers. *Phytochemistry* **19**: 1653-1656.

Doehlert D C., Duke S H. (1983) Specific determination of α -amylase activity in crude plant extracts containing β -amylases. *Plant Physiol.* **71**: 229-234.

Doehlert D C., Knutson C A. (1991) Two classes of starch debranching enzymes from developing maize kernels. *J. Plant Physiol.* **138**: 566-572.

Duwenig E., Steup M., Willmitzer L., Kossmann J. (1997a) Antisense inhibition of cytosolic phosphorylase in potato plants (*Solanum tuberosum* L.) affects tuber sprouting and flower formation with only little impact on carbohydrate metabolism. *Plant J.* **12**: 323 -333.

Duwenig E., Steup M., Kossmann J. (1997b) Induction of genes encoding plastidic phosphorylase from spinach (*Spinacia oleracea* L.) and potato (*Solanum tuberosum* L.) by exogenously supplied carbohydrates in excised leaf discs. *Planta* **203**: 111-120.

Dwelle R B. (1990) Source / sink relationships during tuber growth. *Am. Potato J.* **67**: :829

Edelman J., Singh S P. (1969) Studies on the biochemical basis of physiological processes in the potato tuber. Changes in carbohydrates in the sprouting tuber. *J. Exp. Botany* **17**: 696-702.

Edwards A., Fulton D C., Hylton C M., Jobling S A., Gidley M., Rossner U., Martin C., Smith A M. (1999) A combined reduction in activity of starch synthases II and III of potato has novel effects on the starch of tubers. *Plant J.* **17**: 251-261.

Edwards A et al. (1995) Biochemical and molecular characterisation of a novel starch synthase from potato tubers. *Plant J.* **8**: 283-294.

Egger B., Hampp R. (1996) Activities of enzymes of starch metabolism in developing Norway spruce [*Picea abies* (L.) Karst.] needles. *Trees* **11**: 72-75.

Emes M J., Neuhaus H E. (1997) Metabolism and transport in non-photosynthetic plastids. *J. Exp. Biol.* **48**: 1995 - 2005.

Engels C., Marschner H. (1986) Allocation of photosynthate to individual tubers of *Solanum tuberosum* L. I. Relationship between tuber growth rate and enzyme activities of the starch metabolism. *J. Exp. Botany* **37**: 1795-1803.

FAO Yearbook 1997; Food and Agriculture Organisation of the United Nations, Rome, 1998.

Fiedler G., Pajatsch M., Bock A. (1996) Genetics of a novel starch utilisation pathway present in *Klebsiella oxytoca*. *J. Mol. Biol.* **256**: 279-291.

Fleischmann R D., Adams M D., White O., Clayton R A., Kirkness E F., Kerlavage A R., Bult C J., Tomb J F., Dougherty B A., Merrick J M et al. (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* rd. *Science* **269**: 496-512.

Flipse E., Suurs L., Keetls C.J.A.M., Kossmann J., Jacobsen E., Visser R.G.F. (1996a) Introduction of sense and antisense cDNA for branching enzyme in the amylose-free potato mutant leads to physico-chemical changes in the starch. *Planta* **198**: 340 - 347.

Flipse E., Keetels C.J.A.M., Jacobsen E., Visser R.G.F. (1996b) The dosage effect of the wild type GBSS allele is linear for GBSS activity but not for amylose content: absence of amylose has a distinct influence on the physico-chemical changes in starch. *Theoret. Appl. Genetics* **92**: 121-127.

Fox T.C Geiger D.R. (1984) Effects of decreased night carbon exchange on carbohydrate metabolism in sugar beet source leaves. *Plant Physiol.* **76**: 763-768.

French D (1984) Organisation of starch granules. In RL Whistler, JN Bemiller, EF Parschall, eds, Starch, Chemistry and Technology. Academic Press, New York, pp183-247.

Gaillard T (1987) Starch Availability and Utilisation. In: (T. Gaillard ed.) Starch: Properties and Potential. John Wiley & Sons, Chichester. pp 55-77.

Gallant, D.J., Bouchet, B., Baldwin, P. (1997) Microscopy of starch: evidence for a new level of granule organisation. *Carbohydr. Polym.* **32**: 177-191.

Gana J A., Kalengamaliro N E., Cunningham S M., Volenec J J. (1998) Expression of β -amylase from alfalfa taproots. *Plant Physiol.* **118**: 1495 - 1505.

Gao M., Wanat J., Stinart P S., James M G., Myers A M. (1998) Characteriation of *dull* a maize gene coding for a novel starch synthase. *Plant Cell* **10**: 399-412.

Gaultier N., Foyer C., Huber J L A., Voelker T A., Huber S C. (1993) Effects of elevated sucrose phosphate synthase activity on photosynthesis, assimilate partitioning and growth in tomato (*Lycopersicum esculatum* var UC 82B) *Plant Physiol.* **161**: 535 – 543.

- Geiger D R.** (1976) Effects of translocation and assimilate demand of photosynthesis. *Can. J. Botany* **54**: 2337-2345.
- Geiger M., Stitt M., Geigenberger P.** (1998) Metabolism in slices from growing potato tubers responds differently to addition of sucrose and glucose *Planta* **206**: 234-244.
- Geigenberger P., Stitt M.** (1993) Sucrose synthase catalyses a readily reversible reaction *in vivo* in developing potato tubers and other plant tissues. *Planta* **189**: 329-339.
- Geigenberger P., Merlo L., Reimholz R., Stitt M.** (1994) When a growing potato tuber is detached from its mother plant there is a rapid inhibition of starch synthesis, involving inhibition of ADP-glucose pyrophosphorylase. *Planta* **193**: 486-493.
- Geigenberger P., Reimholz R., Geiger M., Merlo L., Canale V., Stitt M.** (1997) Regulation of sucrose and starch metabolism in potato tubers in response to short-term water deficit. *Planta* **201**: 502-518.
- Geigenberger P., Reimholz R., Dieting U., Sonnewald U., Stitt M.** (1999b) Decreased expression of sucrose phosphate synthase strongly inhibits the water-stress-induced synthesis of sucrose in growing potato tubers. *Plant J.* **19**: 119-129.
- Geigenberger P., Muller-Rober B., Stitt M.** (1999a) Contribution of adenosine 5'-diphosphoglucose pyrophosphorylase to the control of starch synthesis is decreased by water stress in growing potato tubers. *Planta* **209**: 338-345.
- Ghosh P., Meyer C., Remy E., Peterson D., Priess J.** (1992) Cloning expression and nucleotide sequence of a *glgC* gene from an allosteric mutant of *Escherichia coli* B. *Arch. Biochem. Biophys.* **296**: 122- 128.
- Guan H.P., Kuriki T., Sivak M., Priess J.** (1995) Maize branching enzyme catalyses synthesis of glycogen-like polysaccharides in *glgB*-deficient *Escherichia coli*. *Proc. Nat. Acad. Sci. USA* **92**: 964 - 967.
- Guerineau F., Brooks L., Meadows J., Lucy A., Robinson C., Mullineaux P.** (1990) Sulfonamide resistance gene for plant transformation. *Plant Mol. Biol.* **15**: 127 - 136.
- Hargreaves J A., ap Rees T.** (1988) Turnover of starch and sucrose in the roots of *Pisum sativum*. *Phytochemistry* **27**: 1627-1629.

Hausler R E., Schlieben N H., Schulz B., Flugge U I., (1998) Compensation of a decreased triose phosphate / phosphate translocator activity by accelerated starch turnover and glucose transport in transgenic tobacco. *Planta* **204**: 366-376.

Heineke D., Sonnewald U., Bussis D., Gunter G., Leidreiter K., Wilke I., Raschke K., Willmitzer L., Heldt H. W. (1992) Apoplastic expression of yeast-derived invertase in potato. *Plant Physiol.* **100**: 301-308.

Herbers K., Sonnewald U. (1996) Manipulating metabolic partitioning in transgenic plants. *Trends Biotech.* **14**: 198-205.

Hill L., Reimholz R., Schroder R., Nielsen T H., Stitt M. (1996) The onset of sucrose accumulation in cold stored potato tubers is caused by an increased rate of sucrose synthesis and coincides with low levels of hexose phosphates, an activation of sucrose phosphate synthase and the appearance of a new form of amylase. *Plant Cell Env.* **19**: 1223-1237.

Hizukuri S. (1986) Polymodal distribution of the chain lengths of amylopectin and its significance. *Carbohydr. Res.* **147**: 342-347.

Hizukuri S., Tabate S., Nikuni Z. (1970) Studies on starch phosphate. Part 1: estimation of glucose-6-phosphate residues in starch and the presence and the presence of other bound phosphate(s). *Starke* **22**: 338-343.

Hobbs S.L.A., Warkentin T.D., DeLong C.M.O. (1993) Transgene copy number can positively or negatively associated with transgene expression. *Plant Mol. Biol.* **21**: 17 - 26.

Hovenkamp-Hermelink J.H.M., Jacobsen E., Ponstein A.S., Visser R.G.F., Vos-Scheperkeuter G.H., Bijmolt E.W., de Vries J.N., Witholt B., Feenstra W.J. (1987) Isolation of an amylose-free starch mutant of the potato (*Solanum tuberosum* L.). *Theoret. Appl. Genetics* **75**: 217-221.

Hovenkamp-Hermelink J.H.M., De Vries J N., Adamse P., Jacobsen E., Winholt H., Feenstra W J. (1988) Rapid estimation of the amylose/amylopectin ratio in small amounts of tuber and leaf tissue of the potato. *Potato Res.* **31**: 241-246.

International Starch Institute (1998) Typical Composition of Starch Raw Materials. <http://home3.inet.tele.dk/starch/isi/stat/rawmaterial.html>

Isherwood F A. (1973) Starch-sugar interconversion in *Solanum tuberosum* L. *Phytochemistry* **12**: 2579-2591.

Isherwood F A., Burton W G. (1975) The effect of senescence, handling, sprouting, and chemical sprout suppression upon the respiratory quotient of stored potato tubers. *Potato Res.* **18**: 98-104.

Isherwood F A. (1976) Mechanism of starch-sugar interconversion in *Solanum tuberosum*. *Phytochemistry* **15**: 33-41

Isse M.G., Schubert H. (1995) Dissolved starch as an osmotic de-hydrating agent of fruits and vegetables. In *Progress in Plant Polymeric Carbohydrate Research* (Eds Meuser F., Manners D.J., Seikel W.,) Behrs Verlag.

Jacobsen E., Hovenkamp-Hermelink J H M., Krigsheld H T., Nijdam H., Pijnacker I P., Witholt B., Feenstra W J. (1989) Phenotype and genotype of an amylose-free starch mutant of the potato. *Euphytica* **44**: 43 - 48.

James M G., Robertson D S., Meyers A M. (1995) Characterisation of the maize gene *sugary 1*, a determinant of starch composition in kernels. *Plant Cell* **7**: 417-429.

Jane J L., Xu A., Radosavljevic M., Seib P A. (1992) Location of amylose in normal starch granules. I. Susceptibility of amylose and amylopectin to cross-linking reagents. *Cereal Chem.* **69**: 405-409.

Jane J-L., Shen J.J. (1993) Internal structure of the potato starch granule as revealed by chemical gelatinization. *Carbohydr Res.* **247**: 279-290.

Jefferson R. A., Kavanagh T. A., Bevan M. W. (1987) GUS-fusions: β -glucuronidase as a sensitive and versatile gene marker in higher plants. *EMBO J* **6**: 3901-3907.

Jenner C F. (1982) Storage of Starch. In: (F.A Loewus & W. Tanner, eds.) *Encyclopedia of Plant Physiology*. Vol. 13A, Springer-Verlag, Berlin. pp. 700-747.

Jeon B S., Taguchi H., Sakai H., Ohshima T., Wakagi T., Matsuzawa H. (1997) 4- α -glucanotransferase from the hyperthermophilic archaeon *Thermococcus litoralis* -enzyme purification and characterisation and gene cloning, sequencing and expression in *Escherichia coli*. *Eur. J. Biochem.* **248**: 171-178.

Jobling S A., Schwall G P., Westcott R J., Sidebottom C M., Debet M., Gidley M J., Jeffcoat R., Safford R. (1999) A minor form of starch branching enzyme in potato (*Solanum tuberosum* L.) tubers has a major effect on starch structure: cloning and characterisation of multiple forms of SBE A. *Plant J.* **18**: 163-171.

Jones G., Whelan W J. (1969) The action pattern of D-enzyme, a transmaltodextrinylase from potato. *Carbohydr. Res.* **9**: 483-490.

Kakefuda G., Duke S H. (1989) Characterisation of pea chloroplast D-enzyme (4- α -D-glucanotransferase). *Plant Physiol.* **91**: 136-143.

Kakefuda G., Duke S.H., Hostak M.S. (1986) Chloroplast and extrachloroplastic starch degrading enzymes in *Pisum sativum* L. *Planta* **168**: 175 -182.

Kakefuda G., Priess J. (1997) Partial purification and characterisation of a diurnally fluctuating novel endoamylase from *Arabidopsis thaliana* leaves. *Plant Physiol. Biochem.* **35**: 907 - 913.

Kennedy M G H., Isherwood F A. (1975) Activity of phosphorylase in *Solanum tuberosum* L. during low temperature storage. *Phytochemistry* **14**: 667-670.

Killiea S D., Clancy M J. (1978) Properties of potato α -glucosidase. *Phytochemistry* **17**: 1429-1431.

Kitahata S. (1995) In: Enzyme Chemistry and Molecular Biology of Amylases and Related Enzymes, ed., The Amylase Research Society of Japan, Boca Raton (Florida): CRC Press. pp 6-17.

Kleinkopf G E., Westermann D T., Wille M J., Kleinschmidt G D. (1987) Specific gravity of Russet Burbank potatoes. *Am. Potato J.* **64**: 579-587.

Kohli A., Leech M., Vain P., Laurie D A., Christou P. (1998) Transgene organisation in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hotspots. *Proc. Nat. Acad. Sci. USA* **95**: 7203-7208.

Kohli A., Griffiths S., Palacios N., Twyman R M., Vain P., Laurie D A., Christou P. (1999) Molecular characterisation of transforming plasmid rearrangements in transgenic rice reveals a recombination hotspot in the CaMV 35s promoter and confirms the predominance of microhomology recombination. *Plant J.* **17**: 591-601.

Kossmann J., Butcher V., Abel G J W., Duwenig E., Emmermann M., Froberg C., Lloyd J R., Lorbeth R., Springer F., Welsh T., Willmitzer L. (1997) Starch biosynthesis and modification of starch structure in transgenic plants. *Macromolec. Symposia* **120**: 29-38.

Kovacs M I P., Hill R. D. (1974) Starch synthesis in isolated *Phaseolus vulgaris* chloroplasts. *Phytochemistry* **13**: 1335-1339.

Krapp A., Hofmann B., Schafer S., Stitt M. (1993) Regulation of the expression of *rbcS* and other photosynthetic genes by carbohydrates - a mechanism for the sink regulation of photosynthesis. *Plant J.* **3**: 817-828.

Kruger N J., ap Rees T. (1983) Maltose metabolism by pea chloroplasts. *Planta* **158**: 179 - 184.

Kubo A., Fujita N., Harada K., Matsuda T., Satoh H., Nakamura Y. (1999) The starch-debranching enzymes isoamylase and pullulanase are both involved in amylopectin biosynthesis in rice endosperm. *Plant Physiol.* **121**: 399-409.

Kuipers A G J., Soppe W J J., Jacobsen E., Visser R G F. (1994) Field evaluation of transgenic potato plants expressing an antisense granule-bound starch synthase gene: increase of the antisense effect during tuber growth. *Plant Mol. Biol.* **26**: 1759-1773.

Kumar G.N.M., Knowles N.R. (1993) Changes in lipid peroxidation and lipolytic and free-radical scavenging enzyme activities during ageing and sprouting of potato (*Solanum tuberosum* L.) seed-tubers. *Plant Physiol.* **102**: 115-124.

Kunst A., Draeger B., Ziegenhorn J. (1988) D-glucose: UV methods with hexokinase and glucose-6-phosphate dehydrogenase. In: (H.U. Bergmeyer, ed.) *Methods in Enzymatic Analysis*, third edition. Vol. 6. Verlag Chemie, Weinheim. pp.163-171.

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Lao N. T., Schoneveld O., Mould R.M., Hibberd J.M., Gray J., Kavanagh T.A. (1999) An *Arabidopsis* gene encoding a chloroplast-targeted β -amylase. *Plant J.* **20**: 519-527.

Larsson C-L., Khoshnoodi J., Ek B., Rask L., Larsson H. (1998) Molecular cloning and characterisation of starch branching enzyme II from potato. *Plant Mol. Biol.* **37**: 505-511.

Laties G. G. (1962) Controlling influence of thickness on development and type of respiratory activity in potato slices. *Plant Physiol.* **37**: 679-690.

Lauerer M., Saftic D., Quick W P., Labate C., Fichtner K., Schulze E-D., Rodermal S R., Bogorad L., Stitt M. (1993) Decreased ribulose-1,5-bisphosphate carboxylase-oxygenase in transgenic tobacco transformed with 'antisense' *rbcS*. *Planta* **190**: 332-345.

Leidreiter K., Heineke D., Heldt H W., Muller-Rober B., Sonnewald U., Willmitzer L. (1995) Leaf-specific antisense inhibition of starch biosynthesis in transgenic potato plants leads to an increase in photoassimilate export from source leaves during the light period. *Plant Cell Physiol.* **36**: 615-614.

Levi C., Priess J. (1978) Amylopectin degradation in pea chloroplast extracts. *Plant Physiol* **61**: 218 -220.

Li Z., Rahman S., Kosar-Hashemi B., Mouille G., Appels R., Morell K M. (1999) Cloning and characterisation of a gene encoding wheat starch synthase I. *Theoret. Appl. Genetics.* **98**: 1208-1216.

Li Z., Chu X., Mouille G., Yan L., Kosar-Hashemi B., Hey S., Napier J., Shrewry P., Clarke B., Appels R., Morell M K., Rahman S. (1999) The localisation and expression of the class II starch synthases of wheat. *Plant Physiol.* **120**: 1147 – 1155.

Li B., Servaites J. C., Geiger D. R. (1992) Characterisation and subcellular localisation of debranching enzyme and endoamylase from leaves of sugar beet. *Plant Physiol.* **98**: 1277-1284.

Lin T-P., Spilatro S.R., Priess J. (1988a) Subcellular localisation and characterisation of amylases in *Arabidopsis* leaf. *Plant Physiol.* **86**: 251 - 259.

Lin T P., Caspar T., Somerville C., Priess J. (1988b) Isolation and characterisation of a mutant of *Arabidopsis thaliana* (L.) Heynh. Lacking ADPglucose pyrophosphorylase activity. *Plant Physiolb* **86**: 260-265.

Lin T-P., Priess J. (1988) Characterisation of D-enzyme (4- α -glucanotransferase) in *Arabidopsis* leaf. *Plant Physiol.* **86**: 260-265.

Lloyd J R., Bull VJ., Hedley C L., Wand T L., Ring S G. (1996) Determination of the effect of the *r* and *rb* mutations on the structure of amylose and amylopectin in pea (*Pisum sativum* L.). *Carbohydr. Polym.* **29**: 45-49.

Lloyd J R., Landschutze V., Kossmann J. (1999) Simultaneous antisense inhibition of two starch-synthase isoforms in potato tubers leads to accumulation of grossly modified amylopectin. *Biochem J.* **338**: 515-521.

Lorthbeth R., Ritte G., Willmitzer L., Kossmann J. (1998) Inhibition of a starch granule-bound protein leads to modified starch and repression of cold sweetening. *Nature Biotech.* **16**: 473-477.

Lowry O H., Passonneau J V. (1972) A flexible system of enzyme analysis. Academic Press New York.

Manners D.J., Rowe K.L. (1969) Studies on carbohydrate-metabolising enzymes. The α -glucosidase and D-enzyme activity of extracts of carrots and tomatoes. *Carbohydr. Res.* **9**: 441 - 450.

Manners D J. (1985) In: Biochemistry of Storage Carbohydrates in Green Plants. (P. M Det and R.A Dixon, eds.) Academic Press, London pp149-203.

Manners D.J. (1997) Observations on the possible involvement of debranching enzymes in starch biosynthesis. *J. Appl. Glycoscience* **44** : 541 - 546.

Marshall J., Sidebottom C., Debet M., Martin C., Smith A.M. (1996) Identification of the major starch synthase in the soluble fraction of potato tubers. *Plant Cell* **8**: 1121 - 1135.

Martin C., Smith A.M. (1995) Starch biosynthesis. *Plant Cell* **7**: 971-985.

Matheson N K. (1996) The chemical structure of amylose and amylopectin fractions of starch from tobacco leaves during development and diurnally-nocturnally. *Carbohydr. Res.* **282**: 247-262.

Merlo L., Geigenberger P., Hajirazaei M., Stitt M. (1993) Changes of carbohydrates, metabolites and enzyme activities in potato tubers during development and within a single tuber along a stolon-apex gradient. *Plant Physiol.* **142**: 392-402.

Miller S.A., Lipshutz L. (1984) Potato. In, Handbook of Plant Cell Culture, Vol 3, Crop Species, Ed. Ammirato, Evans D.A., Sharp W.R., Yamada Y., Macmillan, Inc. New York. pp 291 - .307.

Mita S., Suzuki-Fujii K., Nakamura K. (1997) Negative regulation in the expression of a sugar-inducible gene in *Arabidopsis thaliana*. A recessive mutation causing enhanced expression of a gene for β -amylase. *Plant Physiol.* **114**: 575-582.

Mohlmann T., Tjaden J., Henrichs G., Quick W.P., Hausler R., Neuhaus H.E. (1997) ADP-glucose drives starch synthesis in isolated maize endosperm amyloplasts: characterisation of starch synthesis and transport properties across amyloplast envelope. *Biochem J.* **324**: 503 - 509.

Moorby J. (1978) The physiology of growth and tuber yield. In; P. M. Harris (ed.), The Potato Crop, The Scientific Basis for Crop Improvement. Chapman and Hall, pp 153-194.

Moreno S., Cardini C E., Tandecarz J S. (1986) α -glucan synthesis on a protein primer, uridine diphosphoglucose:protein transglycosylase I. Separation from starch synthetase and phosphorylase and a study of its properties. *Eur. J. Biochem.* **157**: 539-545.

Morell S., ap Rees T. (1986) Sugar metabolism in developing tubers of *Solanum tuberosum* L. *Phytochemistry* **25**: 1579-1585.

- Mouille, G., Maddelein, M-L., Libessart, N., Talaga, P., Decq, A., Delrue, B., Ball, S.** (1996) Phtoglycogen processing: a mandatory step for starch biosynthesis in plants. *Plant Cell* **8**: 1353-1366
- Mu C., Harn C., Ko Y-T., Singletary G.W., Keeling P.L., Wasserman B.P.** (1994) Association of a 76kDa polypeptide with soluble starch synthase I activity in maize (cv B73) endosperm. *Plant J.* **6**: 151-159.
- Muller-Rober B., Sonnewald U., Willmitzer L.** (1992) Antisense inhibition of the ADP-glucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage proteins. *EMBO J.* **11**: 1229-1238
- Murasheige T., Skoog F.** (1962) A revised medium for the rapid growth and bioassays with tobacco tissue culture. *Physiol. Plantarum* **15**: 473-497.
- Nakamura Y.** (1996) Some properties of starch debranching enzymes and their possible role in amylopectin biosynthesis. *Plant Sci.* **121**: 1- 18.
- Nakamura Y., Vrinten P., hayakawa K., Ikeda J.** (1998) Characterisation of a granule-bound starch synthase isoform found in the pericarp of wheat. *Plant Physiol.* **118**: 451-459.
- Nakano K., Mori H., Fukui T.** (1989) Molecular cloning of cDNA encoding potato amyloplast α -glucan phosphorylase and the structure of its transit peptide. *J. Biochem* **106**: 691-695.
- Nakano K., Fukui T.** (1986) The complete amino acid sequence of potato α -glucan phosphorylase. *J. Biol. Chem.* **261**: 8230-8236.
- Nash L B., Smith O.** (1939) Potato quality II. Relation of mineral nutrition and alterations in light intensity to the cooking quality. *Proc. Am. Soc. Hort. Sci.* **37**: 861- 865.
- Neuhaus H.E., Henrichs G., Scheibe R.** (1993) Characterisation of glucose 6-phosphate incorporation into starch by isolated intact cauliflower-bud amyloplasts. *Plant Physiol.* **101**: 573-578.
- Neuhaus H E., Henrichs G., Scheibe R.** (1995) Starch degradation in intact amyloplasts from isolated cauliflower-bud-amyloplasts. *Plant Physiol.* **64**: 187-192.
- Neuhaus H. E., Stitt M.** (1990); Control analysis of photosynthate partitioning. Impact of reduced activity of ADPglucose pyrophosphorylase or plastid phosphoglucomutase on fluxes to starch and sucrose in *Arabidopsis thaliana* L. Heynh. *Planta* **182**: 445-454.
- Nielsen T.H., Deiting U., Stitt M.** (1997) A β -amylase in potato tubers is induced by storage at low temperature. *Plant Physiol.* **113**: 503-510.
- Nielsen T.H., Wischmann B., Enevoldsen K., Moller B.L.** (1994) Starch phosphorylation in potato-tubers proceeds concurrently with de-novo biosynthesis of starch. *Plant Physiol.* **105**: 111-117.

- Ohad I., Friedberg I., Ne'eman Z., Schramm M.** (1971) Biogenesis and degradation of starch. I. The fate of the amyloplast membranes during maturation and storage of potato tubers. *Plant Physiol.* **47**: 465-477.
- Okita T.W., Greenberg E., Kuhn D.N., Priess J.** (1979) Subcellular localisation of the starch degradative enzymes and biosynthetic enzymes of spinach leaves. *Plant Physiol.* **64**: 187-192.
- Okita T W., Rodriguez J., Priess.** (1981) Biosynthesis of bacterial glycogen - cloning of the glycogen biosynthetic enzyme structural genes of *Escherichia coli*. *J. Biol. Chem.* **256**: 6944.
- Oostergetel G.T., van Bruggen E.F.J.** (1993) The crystalline domains in potato starch are arranged in a helical fashion. *Carbohydr. polym.* **21**: 7-12.
- Oparka K J.** (1986) Phloem unloading in the potato tuber - pathways and sites of ATPase. *Protoplasma* **13**: 201-210.
- Palauqui J C., Elmayan T., Pollien J M., Vaucheret H.** (1997) Systemic acquired silencing : transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. *EMBO J.* **16**: 4738 – 4745.
- Palmer T N., Ryman B E., Whelan W J.** (1976) The action pattern of amylomaltase from *Escherichia coli*. *Eur. J. Biol. Chem.* **69**: 105-115.
- Peach C., Velten J.** (1991) Transgene expression variability (position effect) of CAT and GUS reporter genes driven by linked divergent T-DNA promoters. *Plant Mol. Biol.* **17**: 49 - 60.
- Peat S., Whelan W.J., Rees W.R.** (1956) The enzymatic synthesis and degradation of starch, part 20, the disproportionating enzyme of potato. *J. Chem. Soc.* **56**: 44 - 53.
- Pollock C.J., ap Rees T.** (1975) Activities of enzymes of sugar metabolism in cold stored tubers of *Solanum tuberosum* L. *Phytochemistry* **14**: 1903.
- Pozueta-Romero J., Hrehner M., Viale M., Akawa T.** (1991) ADP-glucose transport by the chloroplast adenylate translocator is linked to starch biosynthesis. *Plant Physiol.* **97**: 1565-1572.
- Priess J., Shen L., Greenberg E., Genter N.** (1966) Biosynthesis of bacterial glycogen *Escherichia coli*. *Biochemistry* **5**: 1833-1845.
- Priess J., Levi C.** (1980) Starch biosynthesis and degradation. In J Priess ed, The Biochemistry of Plnts, Vol 3, Academic Press, New York, pp 428-436.
- Preiss J.** (1988) Biosynthesis of starch and its regulation. In: (J. Priess ed.) The Biochemistry of Plants. Vol. 14, Academic Press, New York. pp. 181-250.
- Priess J., Sivak M.** (1996) Starch synthesis in sinks and sources. In *Photoassimilate Distribution in Plants and Crops* (Zamski, E. and Scahffer A.A, eds.). New York: Dekker, pp. 63-94.
- Radwan M.A., Stocking C.R.** (1957) The isolation and characterisation of sunflower leaf starch. *Am. J. Botany.* **44**: 682-686.

Rahman A., Wong K-S., Jane J-L., Myers A M., James M G. (1998) Characterisation of *Sul* isoamylase, a determinant of storage starch structure in maize. *Plant Physiol.* **117**: 425-435.

Riesmeier J W., Flugge U I., Schulz B., Heineke D., Heldt H W., Willmitzer L., Frommer W B. (1993) Antisense repression of the chloroplast triose phosphate translocator affects carbon partitioning in transgenic potato plants. *Proc. Natl. Acad. Sci. USA* **90**: 6160-6164.

Rodriguez-Lopez M., Baroja-Fernandez E., Zanduetta-Criado A., Pozueta-Romero J. (2000) Adenosine diphosphate glucose pyrophosphatase: A plastidial phosphodiesterase that prevents starch biosynthesis. *Proc. Natl. Acad. Sci. USA* **97**: 8705-8710.

Roeper H., Koch H. (1990) Characterisation of starches for technological uses. In *Progress in Polymeric Carbohydrate Research* (Eds. Meuser F., Manners D., Seikel W) Behrs Verlag. pp 123-130.

Rohde W., Becker D., Salamini F. (1988) Structural analysis of the *waxy* locus from *Hordeum vulgare*. *Nuc Acid Res.* **16**: 7185-7186.

Safford R., Jobling S A., Sidebottom CM., Westcott R J., Cooke D., Tober K J., Strongitharm B H., Russell A., Gidley M J. (1998) Consequences of antisense RNA inhibition of starch branching enzyme activity on properties of potato starch. *Carbohydr. Poly.* **35**: 155-168.

Schachtele C., Steup M. (1986) α -1,4 glucanphosphorylase forms from leaves of spinach (*Spinacia oleracea*) I. In situ localisation by indirect immunofluorescence. *Planta* **167**: 444 - 451.

Schwall G., Safford R., Westcott R. J., Jeffcoat R., Tayal A., Shi Y-C., Gidley M.J., Jobling S.A. (2000) Production of very-high amylose potato starch by inhibition of SBE A and B. *Nature Biotech.* **18**: 551-554.

Schwartz M. (1987) The maltose regulon. In *Escherichia coli* and *Salmonella typhimurum: Cellular and molecular biology*. (Neidhardt F., editor) American Society for Microbiology, Washington D.C. pp. 1482-502.

Shi Y-C., Capitani T., Trzasko P., Jeffcoat R. (1988) Molecular structure of a low-amylopectin starch and high-amylose maize starches. *J. Cereal Starch.* **27**: 289-299.

Shimomura S., Nagai M., Fukui T. (1982) Comparative glucan specificities of the two types of spinach leaf phosphorylase. *J. Biochem.* **64**: 213-225.

Singletary G W., Banisadr R., Keeling P. (1997) Influence of gene dosage on carbohydrate synthesis and enzyme activities in endosperm of starch-deficient mutants of maize. *Plant Physiol.* **113**: 291-304.

Smith A M. (1990a) Enzymes of starch synthesis. In: (P.J. Lea, ed.) *Methods in Plant Biochemistry*. Vol. 3, Academic press, London. pp. 93-102.

Smith A M. (1990b) Evidence that the 'waxy' protein of pea (*Pisum sativum* L.) is not the major starch-granule bound synthase. *Planta* **182**: 599-604.

Smith A M., Denyer K., Martin C. (1997) The synthesis of the starch granule. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **48**: 67 - 87.

Smith A M., Denyer K., Zeeman S C., Edwards A., Martin C. (1999) The synthesis of the starch granule In. *Plant Carbohydrate Biochemistry*. (Eds. Bryant J A., Burrell M M., Kruger N J.,) Bios Scientific Publishers. Oxford. pp79-89.

Sonnewald U., Basner A., Greve B., Steup M. (1995) A second L-type isozyme of potato glucan phosphorylase: cloning, antisense inhibition and expression analysis. *Plant Mol. Biol.* **27**: 567 -576.

Sonnewald U., Haliraezaei M R., Kossmann J, Heyer A., Trethewey R N., Willmitzer L. (1997) Expression of a yeast invertase in the apoplast of potato tubers increases tuber size. *Nature Biotech.* **15**: 794 – 797.

Sonnewald U., Herbers K. (1999) Sugars, far more than just fuel for plant growth. In *Plant Carbohydrate Biochemistry*. (Ed. Bryant J A., Burrell m M B., Kruger N J.,). Bios Scientific Publishers Ltd, Oxford. pp 69-78.

Sowokinos J R., Lulai E C., Knoper J A. (1985) Translucent tissue defects in *Solanum tuberosum* L. 1. Alteration in amyloplast membrane integrity, enzyme activities, sugars and starch content. *Plant Physiol.* **78**: 489-494.

Sowokinos J. (1990) Effect of stress and senescence on carbon partitioning in stored potatoes. *Am. Potato J* **67**:849 – 857.

Sowokinos J. (1990b) Stress-induced alterations in carbohydrate metabolism. In *The Molecular and Cellular Biology of the Potato*. Ed. Vayda M E., Park W D., CAB International Wallingford pp 137 – 158.

St-Pierre B., Brisson N. (1995) Induction of the plastidic starch phosphorylase gene in potato storage sink tissue. Effect of sucrose and evidence for coordinated regulation of phosphorylase and starch biosynthetic genes. *Planta* **195**: 339 - 344.

Stam M., de Bruin R., Kenter S., van der Hoorn R A L., van Blokland R., Mol J N M., Kooter J M. (1997) Post-transcriptional silencing of chalcone synthase in *Petunia* by inverted transgene repeats. *Plant J.* **12**: 63-82.

Stark D.M., Timmerman K.P., Barry G.F., PriessJ., Kishore G.M. (1992) Regulation of the amount of starch in plant tissues by ADP-glucose pyrophosphorylase. *Science* **258**: 287-292.

Steup M., Latzko E. (1979) Intracellular localisation of phosphorylases in spinach and pea leaves. *Planta* **145**: 69-75.

Steup M., Robenek H., Melkonian M. (1983) *In vitro* degradation of starch granules isolated from spinach chloroplasts. *Planta* **158**: 428-436.

Steup M. (1988) Starch degradation. In: J Priess ed, *The Biochemistry of Plants*, Vol 14. Academic Press, New York, pp 255 - 289.

Steup M., Schachtele C. (1981) Mode of glucan degradation by purified phosphorylase forms from Spinach leaves. *Planta* **153**: 351-361.

Steup M., Yang Y., Greve B., Weiler E.W. (1991) Characterisation of a heteroglycan from higher plants. In *Recent Advances in Phloem Transport and Assimilate Compartmentation* (Bonnemain, J.L., Delrot, S., Lucas, W.L., Dainty, J., eds.) Nantes: Ouest Editions, pp. 224 - 232.

Stitt M., Bulpin P V., ap Rees T. (1978) Pathway of starch breakdown in photosynthetic tissues of *Pisum sativum* *Biochem. Biophys. Acta* **544**: 200-214.

Stitt M., Steup M. (1985) Starch and sucrose degradation. In: Higher plant cell respiration. Encyclopaedia of plant physiology, N.S., vol. 18. (Douce R., Day D.A., eds) Springer, Berlin Heidelberg New York. pp 345 - 390.

Stitt M. (1997) The flux of carbon between the chloroplast and cytoplasm. In: Dennis D.T., Turpin D.H., Lefebvre D.D., Layzell D.B (eds) *Plant Metabolism*. Addison Wesley Longman, London, pp 382-400.

Sugunuma T., Setoguchi S., Fujimoto S., Nagahama T. (1991) Analysis for the characteristic action of D-enzyme from sweet potato in terms of subsite theory. *Carbohydr Res.* **212**: 201 - 212.

Sullivan T.D., Kaneko. (1995) The maize *brittle 1* gene encodes amyloplast membrane polypeptides. *Planta* **196**: 477-484.

Sun C., Sathish P., Ahlandsberg S., Jansson C. (1999) Analyses of isoamylase gene activity in wild-type barley indicate its involvement in starch synthesis. *Plant Mol. Biol.* **40**: 431 - 443.

Sun Z., Henson S.H. (1990) Degradation of native starch granules by barley α -glucosidases. *Plant Physiol.* **94**: 320 -327.

Sun H., Duke S.H., Henson C. (1995) The role of pea chloroplast α -glucosidase in transitory starch degradation. *Plant Physiol.* **108**: 211 - 217.

Sweetlove L J. (1996) The control of ADPglucose over starch synthesis in potato (*Solanum tuberosum* L.) tubers. PhD thesis, University of Cambridge.

Sweetlove L J, Kossmann J., Riesmeier J W., Trethewey R N., Hill S A. (1998) The control of source to sink carbon flux during tuber development in potato. *Plant J.* **15**: 697-706.

Sweetlove L.J., Burrell M.M., ap Rees T. (1996a) Characterisation of transgenic potato (*Solanum tuberosum*) tubers with increased ADPglucose pyrophosphorylase. *Biochem J.* **20**: 487 - 49.

Sweetlove L.J., Burrell M.M., ap Rees T. (1996b) Starch metabolism in tubers of transgenic potato (*Solanum tuberosum*) with increased ADPglucose pyrophosphorylase. *Biochem J.* **320**: 493 - 498.

Sweetlove L. J., Muller-Rober B., Willmitzer L., Hill S. A. (1999) The contribution of adenosine 5'-diphosphoglucose pyrophosphorylase to the control of starch synthesis in potato tubers. *Planta* **209**: 330-337.

Sweetlove L. J., Hill S. A. (2000) Source metabolism dominates the control of source to sink carbon flux in tuberising potato plants throughout the diurnal cycle under a range of environmental conditions. *Plant Cell Env.* **23**: 523-529.

Takaha T., Yanase M., Okada S., Smith S.M. (1993) Disproportionating enzyme (4 α -glucanotransferase; EC 2.4.1.25) of potato. *J. Biol. Chem.* **268**: 1391 - 1396.

Takaha T. (1996) Isolation of Disproportionating enzyme (ECC 2.4.1.25) from potato and investigation of its role in starch metabolism. Ph.D. Thesis, University of Edinburgh.

Takaha T., Yanase M., Takata H., Okada S., Smith S.M. (1996) Potato D-enzyme catalyses a novel cyclisation of amylose to produce cycloamylose, a novel cyclic glucan. *J. Biol. Chem.* **271**: 29012 - 29018.

Takaha T., Smith S.M. (1998b) The functions of 4- α -glucanotransferases and their use for the production of cyclic glucans. *Biotech. Gen. Eng. Rev.* **16**:

Takaha T., Yanase M., Takata H., Okada S., Smith S.M. (1998a) Cyclic glucans produced by the intramolecular transglycosylation activity of potato D-enzyme on amylopectin. *Biochem. Biophys. Res. Comm.* **247**: 493 - 497.

Takaha T., Critchley J., Okada S., Smith S. M. (1998c) Normal starch content and composition in tubers of antisense potato plants lacking D-enzyme (4- α -glucanotransferase). *Planta* **205**: 445-451.

Takeda T., Hizukuri S. (1982) Location of phosphate groups in potato amylopectin. *Carbohydr. Res.* **102**: 321 - 327.

Takeda Y., Hizukuri S. (1987) Structures of rice amylopectins with high and low affinities for iodine. *Carbohydr. Res.* **168**: 79-88.

Takeda Y., Shirasaka K., Hizukuri S. (1984) Examination of the purity and structure of amylose by gel-permeation chromatography. *Carbohydr. Res.* **132**: 83-94.

Takeda Y., Guan H-P., Priess J. (1993) Branching of amylose by the branching isozymes of maize endosperm. *Carbohydr. Res.* **240**: 253-263.

Tatge H., Marshall J., Martin C., Edwards E.A., Smith A.M. (1999) Evidence that amylose synthesis occurs within the matrix of the starch granule in potato tubers. *Plant Cell Env.* **22**: 543-550.

Thorbjornsen T., Villand P., Denyer K., Olsen O-A., Smith A.M. (1996) Distinct isoforms of ADPglucose pyrophosphotylase occur inside and outside the amyloplasts in barley endosperm. *Plant J.* **10**: 243-250

Tjaden J., Mohlmann T., Kampfenkel K., Henrichs G., Neuhaus H E. (1998) Altered plastidic ATP/ADP-transporter activity influences potato (*Solanum tuberosum* L.) tuber morphology, yield and composition of tuber starch. *Plant J.* **16**: 531-540.

Tomlinson K L., Lloyd J R., Smith A M. (1997) Importance of isoforms of starch branching-enzyme in determining the structure of starch in pea leaves. *Plant J.* **11**: 31 - 43.

Tomlinson K L., Craig J., Smith A M. 1998; Major differences in isoform composition of starch synthase between leaves and embryos of pea (*Pisum sativum* L.). *Planta* **204**: 86 - 92.

Trethewey R., ap Rees T. (1994a) A mutant of *Arabidopsis thaliana* lacking the ability to transport glucose across the chloroplast envelope. *Biochem J.* **301**: 449-454.

Trethewey R N., Riesmeier J W., Willmitzer L., Stitt M., Geigenberger P. (1999) Tuber-specific expression of a yeast invertase and a bacterial glucokinase in potato leads to an activation of sucrose phosphate synthase and the creation of a sucrose futile cycle. *Planta* **208**: 227-238.

Van Eck H.J., Jacobs J.M.E., Stam P., Ton J., Stiekama W.J., Jacobsen E. (1994) Multiple alleles for tuber shape in diploid potato detected by qualitative and quantitative genetic analysis using RFLPs. *Genetics* **137**: 303 - 309.

Van de Wal M., et al, (1998) Amylose is synthesised *in vitro* by extension of and cleavage from amylopectin. *J. Biol. Chem.* **273**: 22232-22240.

Veramendi J., Willmitzer L., Trethewey R N. (1999) In vitro grown potato microtubers are a suitable system for the study of primary carbohydrate metabolism. *Plant Physiol. Biochem.* **37**: 693-697.

von Sch—ele C ., Svensson G., Rasmusson J. (1937) Die Bestimmung des Stärkegehalts und der Trockensubstanz der Kartoffel mit Hilfe des spezifischen Gewichts. *Landwirtschaftlichen Versuchsstationen Bd.* **127**: 67-96.

Waigh T A., Jankins P J., Donald A M. (1996) Quantification of water in carbohydrate lamellae using SANS. *Faraday Discussions* **103**: 325-337.

Waigh T A., Hopkinson I., Donald A M. (1997) An analysis of the structure of native starch granules with x-ray microfocus diffraction. *Macromolecules* **30**: 3813-3820.

Waigh T A., Perry P., Reikel C., Gidley M J., Donald A M. (1998) Chiral side-chain liquid-crystalline polymeric properties of starch. *Macromolecules*. **31**: 7980-7984.

Wallis J.G., Dziewanowska K., Guerra D.J. (1986) Genetic transformation with the *su1* gene; a highly efficient selectable marker for *Solanum tuberosum* L. cv. Russet Burbank. *Molec. Breeding* **2**: 283 - 290.

Wardlaw I F. (1990) The control of carbon partitioning in plants. *Ann. Rev. Plant Physiol.* **116**: 341-381.

Weatherwax P. (1922) A rare carbohydrate in waxy maize. *Genetics* **7**: 568-572.

Willmitzer L., Kossmann J., Muller-Rober B., von Schaeven A., Sonnewald U. (1993) Approaches to influence the partitioning and metabolism of carbohydrates in transgenic plants. In Control of Plant Gene Expression. CRC Press.

Wirtz W., Stitt M., Heldt H.W. (1980) Enzymatic determination of metabolites in the subcellular compartments of spinach protoplasts. *Plant Physiol.* **66**: 187 -193.

Wischmann B., Nielsen T H., Moller B L. (1999) In vitro biosynthesis of phosphorylated starch in intact potato amyloplasts. *Plant Physiol.* **119**: 455-462.

Wise Jr E M., Abou-Donia M M. (1975) Sulfonamide resistance mechanism in *Escherichia coli*: R Plasmids can determine sulfonamide-resistant dihydropteroate synthases. *Proc. Nat. Acad. Sci. USA* **72**: 2621-2625.

Wolters A-M A., Janssen E.M., Rozeboom-Schippers G.M., Jacobsen E., Visser R.G.F. (1998) Composition of endogenous alleles can influence the level of antisense inhibition of granule-bound starch synthase gene expression in tetraploid potato plants. *Molec. Breeding* **4**: 343-358.

Worrell A C., Bruneau J M., Summerfelt K., Boersig M., Volker T A. (1991) Expression of a maize sucrose phosphate synthase in tomato affects leaf carbohydrate partitioning. *Plant Cell* **3**: 1121 – 1130.

Xoconostle- Cazares B., Xiang Y., Ruiz-Medrano R., Wang H-L., Monzer J., Yoo B-C., MacFarland K C., Franceschi V R., Lucas W J. (1999) Plant viral movement protein potentiates transport of mRNA into the phloem. *Science* **283**: 94 – 98.

Yandell B. S., Najar., Wheeler R., Tibbitts T.W. (1988) Modelling the effects of light, carbon dioxide and temperature on the growth of potato. *Crop Science* **28**: 811-818.

Yang B-Z., Dind J-H., Enghild J.J., Bao Y., Chen Y-T. (1992) Molecular cloning and nucleotide sequence of cDNA encoding human muscle glycogen debranching enzyme. *J. Biol. Chem.* **267** : 9294 - 9299.

Yang Y., Steup M. (1990) Polysaccharide fraction from higher plants which strongly interacts with the cytosolic phosphorylase isozyme. I. Isolation and characterisation. *Plant Physiol.* **94**: 960 -969.

Yoshio N., Maeda I., Tangiguchi H., Nakamura M. (1986) Purification and properties of D-enzyme from malted barley *J. Jpn. Soc. Starch Sci.* **33**: 244-252.

Zeeman S C., Umemoto T., Lue W L., Au-Leung P., Martin C., Smith A., Chen J. (1998a); A mutant of *Arabidopsis* lacking a chloroplastic isoamylase accumulates both starch and phytoglycogen. *Plant Cell* **10**: 1699-1712.

Zeeman S. C. Northrop F., Smith A.M., ap Rees T. (1998b) A starch-accumulating mutant of *Arabidopsis thaliana* deficient in a chloroplastic starch-hydrolysing enzyme. *Plant J.* **15**: 357-365.

Zhu Z., Hylton C M., Rossner U., Smith A M. (1998) Characterisation of starch-debranching enzymes in pea embryos. *Plant Physiol.* **118**: 581-590.

Ziegler P., Beck E. (1986) Exoamylase activity in vacuoles isolated from pea and wheat leaf protoplast *Plant Physiol.* **82**: 1119-1121.

Ziegler P., Beck E. (1989) A phosphate translocator is present in photosynthetically inactive plastids of dark-germinated pinyon seedlings. *Plant Physiol.* **111**: (Suppl.): 108.

Zrenner R., Salanoubat M., Willmitzer L., Sonnewald U. (1995) Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (*Solanum tuberosum* L.). *Plant J.* **7**: 97 - 107.

Zrenner R., Krause K-P., Apel P., Sonnewald U. (1996) Reduction of the cytosolic fructose 1,6-bisphosphatase in transgenic potato plants limits photosynthetic sucrose biosynthesis with no impact on plant growth and tuber yield. *Plant J.* **9**: 671-681.